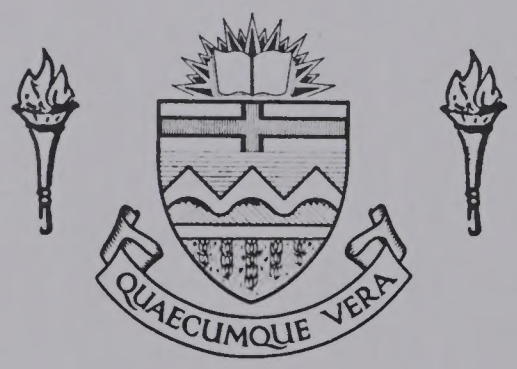

THE UNIVERSITY OF ALBERTA

THE EFFECT OF GAMMA-IRRADIATION ON SULFUR CONTAINING COMPONENTS OF SKIMMILK POWDER

By

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

THE EFFECT OF GAMMA-IRRADIATION ON SULFUR CONTAINING COMPONENTS OF SKIMMILK POWDER

submitted by Hua Hsu in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The effect of gamma-irradiation on the off-flavor development in skimmilk powders was studied by analysis of the sulfur constituents of the powder. Irradiation of commercial high heat spray-dried and laboratory freeze-dried skimmilk powders resulted in an increase of the free, masked and total -SH groups which was assumed to be caused by the scission of disulfide bonds and by the unfolding of milk proteins.

The increase in the -SH groups for laboratory prepared skimmilk powder was in per cent, 15.3 to 69.2 for free, 6.4 to 9.3 for masked and 6.4 to 29.3 for total -SH groups at irradiation doses of 0.4, 0.8, 1.2 and 1.6 Mrad. Measurable amounts of free -SH groups were not detected in either the control, non-irradiated powder, nor in the powder exposed to a 0.4 Mrad dose. The percentage increase of -SH groups in the commercial skimmilk powder was 2 to 20 for free, 7.3 to 9.4 for masked and 12.8 to 32.3 for the total -SH groups. The initial content of free -SH groups averaged 18.5 mM/100 g of skimmilk powder. In both laboratory and commercially prepared skimmilk powders the increase in -SH groups was related to the irradiation dose applied.

Both free radical scavengers, ascorbic acid and ascorbyl palmitate, have shown a protective effect on milk proteins when present during irradiation. For additions of 2.72 to 23.8 mM of ascorbic acid/100 g of laboratory skimmilk powder, the protective effect at an irradiation dose of 0.4 Mrad was reflected in a

decrease of the total -SH group of from 2.4 to 16.5 per cent compared with the control. Hence, the free and masked -SH groups also decreased. Ascorbyl palmitate at an irradiation dose of 0.4 Mrad and similar concentrations, induced a protective effect amounting to a decrease of total -SH group from 5.5 to 29.9 per cent. At an irradiation dose of 1.6 Mrad similar protection was obtained. Therefore, as a scavenger additive ascorbyl palmitate was more effective than ascorbic acid.

The application of labelled $^{14}C_1$ - ascorbic acid has shown that during irradiation a portion of the additive is also degraded into compounds other than carbonyls.

Sulfur-containing volatiles present in irradiated commercial skimmilk powder were determined to be ethyl-, n-propyl-, n-butyl-, sec-butyl- and allyl-mercaptans and diallyl sulfide. Ethyl mercaptan, n-propyl- and allyl- mercaptans were not found in non-irradiated sample and the other compounds were present only in trace amounts.

The volatiles not containing sulfur were also separated and tentatively identified to be aldehydes, ketones, and lower members of the alkanoic acids, which finding supports the assumption that the residual amounts of 0.6 per cent of lipid in skimmilk powder also contributed to the development of off-flavors in irradiated milk powder.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES *PAGE*

I. INTRODUCTION

Extensive investigation to assess the effectiveness of ionizing irradiation in the preservation of a wide range of foods has shown that this new method of food preservation is not an unqualified success. For a considerable number of foods, including dairy products, the irradiation dose required to effect the destruction of contaminating microorganisms to ensure preservation is sufficient to cause serious defects in the flavor and appearance of the food.

In milk irradiation induced defects are reported to develop at relatively low doses of less than 100 Krad, which in fact are only "pasteurizing" treatments. It is recognized that these side effects of irradiation are principally caused indirectly through radiolysis of the water content of milk which generates highly reactive peroxides and free radicals. Consequently, a food low in water content, such as skimmilk powder, that normally has a water content less than 5 per cent, should be less sensitive to irradiation flavor defects.

Unpublished data (Wood, 1968) obtained in the irradiation of skimmilk powder inoculated with Salmonella reported the development of an "irradiation" or "wet dog" flavor when the powder was recombined with water. This flavor was similar to that produced by the irradiation of liquid skimmilk. The apparent lack

of published information on the chemical changes resulting from the irradiation of skimmilk powder and, the observation that the odor and flavor of the recombined skimmilk resembled volatile sulfur compounds, prompted this investigation of the volatile sulfur compounds of irradiated skimmilk powders. The effectiveness of the irradiation of packaged skimmilk powder in eliminating Salmonella contamination of the dairy product, which is a serious food poisoning problem, encouraged a limited investigation of irradiated flavor desensitizing additives.

II. REVIEW OF LITERATURE

A. Preservation of Food by Ionizing Irradiation

1. General aspects

Research directed toward the use of ionizing irradiations for the preservation of foods began in 1943 with the use of X-rays as a method of improving the keeping quality of hamburgers (Barratt, 1967). In 1954, the first application of Cobalt-60 as a means of controlling the sprouting of potatoes was reported. Because of the nature of ionizing irradiations their use in the commercial sterilization of foods has placed them in the same category as that of a food additive, which must be approved by the Food and Drug Division of the Department of Health and Welfare in Canada, or the Food and Drug Administration in the United States. It has been officially approved by the Food and Drug Administration in the United States for bacon, wheat and wheat flour preservation, insect disinfection, sprout inhibition of white potato and the sterilization of some packaging materials. However, the regulation permitting the irradiation sterilization of bacon was revoked in August 1968. In Canada the Food and Drug Directorate permits the use of Gamma-irradiation as an inhibitor of sprouting for both potatoes and onions.

Goldblith (1970) proposed three categories to describe food irradiation treatments in increasing order of dosage: radicidation, radurization and radappertization. Treatments of foods at a

dosage of less than 0.75 Mrad* have been recommended for the removal of Salmonellae in frozen chicken, in eggs and in animal feed stuffs (Licciardello, 1964; Grim and Goldblith, 1965; Thornley, 1963). Studies in the Netherlands have shown that animal feedstuffs treated at this dosage were wholesome and free of toxic substances. Such treatment was classified as radicidation. Radurization of foods refers to a process for extending the storage life of foods through the use of a sub-sterilization dose in order to destroy the bulk of non-spore formers. This treatment has been successfully applied to fin fish, like cod and haddock, shrimp, and fruits, such as strawberries and papayas. The simple fact that dosages of one-tenth to one-fifth of that needed for sterilization are used in radurization processes there is concommitantly a much lower production of secondary side effects. Radappertization refers to the irradiation of foods at low temperatures. Highly acceptable products have been produced by this method in studies carried out by the US Army at Natick Laboratories, where it has been shown that not only irradiation sterilized ham and bacon were highly acceptable products, but also chicken, pork, beef, shrimp and codfish cakes as well. Unfortunately, irradiation has not yet been successfully applied to dairy products.

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* 1 rep (roentgen equivalent physical)
= an energy absorption of 93 ergs per cubic centimeter of tissue.

1 rad = the absorption of 100 ergs per g of material.

The rad is approximately 1.1 times larger than the rep reported in the literature.

2. Milk preservation

Milk preservation by ionizing irradiation was one of the earliest suggested uses for irradiation. Considerable research had been done by 1959 with no definite breakthrough. A review of the literature by Veringa (1962) of the Netherlands Dairy Research Institute showed no real progress in developing a satisfactory irradiation based process for milk and milk products.

When irradiation alone is used to sterilize fluid milk, a dose of about 1.86 Mrad is generally considered necessary under aerobic conditions (Brownell et al. 1953). Work at the University of Miami (Read et al. 1958) indicated that for evaporated milk 2.79 Mrad is well above the dose needed for sterilization. Goldblith and Proctor (1956) indicated a dose requirement of 1.5 to 4.0 Mrep. An earlier report by Dunn et al. (1948), from Massachusetts Institute of Technology (MIT), indicated 1 Mrep or less to be a sterilizing dose. Freeman (1959) achieved apparent sterility immediately after irradiation with 0.25 to 0.75 Mrad, but growth of microorganisms occurred after 11 days storage at room temperature. However, he reported no growth of microorganisms during storage of samples irradiated at 1 to 1.25 Mrad.

The irradiation dose required for milk pasteurization is considerably lower than that required for sterilization (Proctor and Goldblith (1951). Gaden et al. (1951) reported that 0.146 Mrep of X-radiation destroyed 99.9 percent of microorganisms present in raw whole milk, while Dluzewski and Pijanowski (1964) obtained a

99.9 per cent decrease in the coliform organisms with 0.120 Mrad of Co^{60} irradiation. As pointed out by Proctor and Goldblith (1951) the order of susceptibility of bacterial species to irradiation is in general the same as that to heat. A dose of 0.1 Mrep eliminates Escherichia coli from ilk, while 2 Mrep is required for Clostridium sporogens spores. A dose of 0.054 Mrep at 5°C was sufficient to destroy the total bacteria, the caseolytes, and coliforms, to the extent of 98.6, 100, and 100 per cent, respectively. However, at higher temperature, 25°C, the bacteria multiplied faster than the irradiation could destroy them (Worseck 1961).

Irradiation doses required for complete destruction of bacteria are lower by a factor of then than those required for the inactivation of milk enzymes (Proctor and Goldblith, 1951). Approximately 10 to 25 Mrad were necessary to inactivate completely the phosphatase in raw whole milk. Catalase and peroxidase have been reported to be more irradiation sensitive than phosphatase (Tsugo and Hayashi, 1961). As found by O'Meara (1952) the effect of irradiation on an enzyme is indirect with water free radicals playing an important role. On the other hand, compounds present in solution may protect the enzyme against irradiation. Hence, the irradiation resistance of an enzyme in milk is considerably greater than that of an enzyme in pure solution.

Irradiation causes considerable losses of vitamins in milk. A Co^{60} irradiation of either raw or pasteurized milk at a dose of 0.48 Mrad destroyed 100 per cent of the ascorbic acid, 40

per cent of the carotene, 70 per cent of the vitamin A, and 60 per cent of tocopherol (Kung et al. 1953). The thiamine destruction in pasteurized milk was up to 35 per cent at 0.15 Mrad, in condensed milk up to 85 per cent at 2 Mrad, and in powdered milk 100 per cent destruction was reported using doses of 2.79 or 5.58 Mrad.

However, it is not the vitamin destruction which forms the principal obstruction to commercial application of radiation sterilization. Side effects, chemical reactions resulting in off-flavor and off-odors are the major disadvantages of irradiation which makes the milk unpalatable. In addition, there is a denaturation and interaction of milk proteins which are reflected by a change of protein solubility, viscosity, electrophoretic mobility, tendency to gel, increase in rennet coagulation time, and molecular weight.

B. Irradiation Effects on Milk Proteins

1. Off-flavor development

Milk is particularly sensitive to ionizing irradiation and develops off-flavors at 1 per cent of the dose required for sterilization. The type of irradiation used, X-, gamma, or cathode rays, have little effect on the dose at which off-flavor occurs (Goldblith and Proctor 1954). The threshold dose at which cathode rays produce off-flavors was found to vary from 7 to 25 x 10^{-3} Mrep, depending on the type of milk, dry or fluid, whole or skim, raw or pasteurized, homogenized or not, and evaporated or not. Off-flavor production increased with increasing moisture or fat content, but decreased with the increasing content of total solids (Bierman et al. 1956).

As evaluated by Day et al. (1957) the flavors and odors of irradiated skimmilk systems at 2 Mrep were as follows; skimmilk - malty and caramel-like, nondialysable fraction of skimmilk - putrid, cooked cabbage and burnt protein; diffusable moiety - caramel-like; sodium caseinate sols - putrid, cooked cabbage and burnt protein, and finally lactose in 5 per cent solution - sour with no odor. In the same study the flavor threshold values were determined for a few carbonyl compounds, such as acetaldehyde, acetone and butanone and compared with those of sulfur compounds found in irradiated milk. The results confirmed the extreme flavor potency of the sulfur compounds. Their threshold values were in the realm of parts per billion and their flavor quality ranged from putrid, burnt for methyl disulfide, malty, cowy for methyl sulfide and putrid cooked cabbage-like for methyl mercaptan.

Of the volatile carbonyl compounds produced in skimmilk by irradiation, acetaldehyde seemed to be the only one of malty flavor significance. The other carbonyls, such as acetone, butanone n-hexanal were present well below their threshold values.

In addition to these flavors a chalky off-flavor of milk was reported when using 2 Mrep of gamma-radiation, which was attributed partly to peroxides formed from the more saturated components, while an oxidized rancid flavor was reported and claimed to be derived from the unsaturated fraction of butter fat. The off-flavor developed from milk fat using 4.5 Mrep Co^{60} contained a candle-like odor which was attributed to carbonyls with medium to long carbon

chains, or derivatives of acids containing a vinyl group and branched chains (Wertheim et al. 1957).

Recovery of flavor volatiles from milk by a low temperature reduced pressure distillation technique yielded further information on the nature of some off-flavors present in milk (Day et al. 1957). The strong sulfur-type odor obtained in a cold trap could be converted by sodium hydroxide to an amine-type odor. Acidification of the basic solution again produced the sulfur-type odor. Mercuric chloride irreversibly destroyed most of the odor of the distillate. Acidified aqueous 2,4 - dinitrophenylhydrazine (2,4 DNPH) removed part of the malty character. While the distillation technique removed most of the off-flavors from irradiated milk, such as the malty, putrid and burnt protein off-flavors, the caramel-like defect was not volatile and remained with the residual milk. This observation appeared in part to support the contention of Wertheim et al. (1956) that removal of volatiles by vacuum treatment, during irradiation, is effective in preventing off-flavor and odor development in milk. The non-volatile caramelized flavor is not a unique problem of irradiation. As pointed out by Wertheim et al. (1956), this flavor is associated with non-enzymatic browning of milk, and involved reactions between amino compounds and milk carbonyls.

Identification of volatile components of skimmilk irradiated at 2 Mrep, using gas chromatography and mass spectrometry, revealed the presence of methyl- and ethyl alcohols, beside the previously determined mercaptans, sulfides and carbonyls (Day et al. 1957). Through the use of a low temperature and a reduced distillation

technique with six consecutive cold traps made up as follows: two wet ice, two ethanol and dry ice, and two liquid nitrogen, they collected mercaptans, sulfides and carbonyls in the first three traps, methyl alcohol in the fourth and ethyl alcohol in the fifth trap. Simultaneously, it was determined that like acetone and butanone these alcohols were of minor importance as off-flavors and odor contributors. On the other hand, methyl mercaptan appeared to be the major off-flavor component of the sodium caseinate sols irradiated at 2 Mrep. Methyl sulfide and acetaldehyde were of secondary importance. As in the case of skimmilk the methyl disulfide could not be identified, but judging from the odor and the ease with which methyl mercaptan could be oxidized the presence of disulfide was suggested by Day et al. (1957).

The presence of acetaldehyde and methyl sulfide in irradiated skimmilk and sodium caseinate sols suggested that milk proteins were their major precursors. The source of the sulfur compounds appeared to be the sulfur-containing amino acids while acetaldehyde has been revealed as an irradiation product of alanine (Sharpless et al. 1955). As stated by Day et al. (1957) the convincing explanation for the formation of the identified compounds might involve the production of free radicals involving water, air and amino acids. In two irradiated samples, skimmilk and sodium caseinate, methyl alcohol was detected only in the former and methyl mercaptan only in the latter. The two compounds were probably derived from the same methyl radical, but the difference in their occurrence seemed

to depend on the availability of free sulfhydryl radicals. Since the quantity of radicals depends on the available -SH groups, it appears that the skimmilk lacks this group as a precursor. As observed by Patton and Josephson (1949) in a milk sample undergoing heat induced browning -SH groups disappear. Hence, it appears that the off-flavor development in irradiated skimmilk might be dependent on a previous heat treatment of the milk sample, and/or by the heat generated in the sample during irradiation.

2. Off-flavors induced by heat treatment

Of all the processes to which milk is exposed, heating is probably the most fundamental. Pasteurization, heat sterilization, preheating, superheating are the well known processing methods. Such treatment of milk is not without effect on its flavor and in general the more intensive the heat treatment the more pronounced the flavor change that is detected. These changes could be explained fairly well by the heat denaturation of the milk serum proteins.

In the native state the milk serum proteins have a definite coiled configuration maintained by hydrogen bonding, hydration, etc. When these proteins are exposed to heat above a critical temperature the native configuration is disrupted and the properties of the proteins are altered. Consequently a remarkable change of solubility and reactivity of their sulfhydryl groups are observed. The sulfhydryl groups are apparently occluded or masked in the native protein in a manner that keeps them unreactive. By heat treatment the protein

uncoils and the groups become more accessible. In such a state they are chemically more reactive. The activation of -SH groups becomes evident about 75°C. The evolution of volatile sulfides and the appearance of cooked flavor in heated milk parallels this activation. In addition, the heat treatment of the serum proteins could also induce a loss of their capacity to form a normal cream layer. Overheating during pasteurization of milk initiates denaturation of the globulins which are responsible for normal creaming, since they support the formation of clusters among the rising fat globules. The cream layer formed in such milk is shallow and its flavor is termed burnt and cabbagey.

In recent years considerable attention has been focused on detecting and measuring volatile and non-volatile sulfhydryl components of heated milk and milk products. The terms "reactive" and "active" are used to differentiate the heat revealed - SH groups from the total - SH concentration in unheated milk. The volatile portion of the reactive -SH groups is usually determined by a distillation in a stream of nitrogen and the use of a solution of zinc acetate as a trap and this portion is expressed as H_2S (Townley and Gould 1943). The non-volatile portion of the heat revealed -SH compounds is measured by the thiamine disulfide (TDS) method of Harland and Ashworth (1945).

The data of Boyd and Gould (1957) offer evidence that the production of volatile -SH compounds by heat is a two-step process: first, the production of precursors which reduce TDS, and, second,

the decomposition of a portion of these precursors to permit evolution of the sulfur-containing volatiles. As shown earlier by Zweig and Block (1953) the effect of heating on -SH groups causes, at the beginning of heating, a slight initial increase in reducible -SH groups and then with increased heating a decrease occurs. Only a slight change in -SH concentration was observed up to a temperature of 58 - 69°C but above this temperature the -SH concentration decreased rapidly. The break in the plotted curve representing the concentration dependence of -SH on temperature was designated the "critical temperature". As stated by the same authors the variability of the critical temperature from batch to batch of milk may be related to the commercial experience that different samples of milk should be heated to different temperatures with variable holding times to achieve similar end products. Similarly, it was found that the critical temperatures for skimmilk are within the range of 71 - 77°C. It was also apparent that the holding time prior to drying at temperatures above that of the critical temperature, had a pronounced effect on the loss of -SH groups. The milk held at 76.6°C for 5 minutes prior to spray drying, contained 0.22 m.eq. -SH groups per 100 g of powder, and when the holding time was lengthened to 20 minutes this value fell to 0.16 m.eq.

The effect of flash heat treatment on the -SH groups revealed a variable and considerable loss of -SH groups at the higher temperatures, such as 120 - 150°C. This was explained as being a reflection of the fact that very slight variations in the

holding time in this type of heat treatment, could result in significant differences in the physical properties of the final product (Zweig and Block 1953).

As found by Hutton and Patton (1952) all the -SH groups of milk are in the whey proteins such as β-lactoglobulin and partly in the fat globule membrane protein. Furthermore, they demonstrated that a straight line could be obtained when the log of the concentration of -SH groups was plotted against that of holding time. Hence, the loss of -SH groups found on heating milk above the critical temperature should be a first order reaction reflecting the folding-unfolding changes of β-lactoglobulin and other proteins. In addition, the cooked flavor of the heated milk was reported to be associated with the decomposition of the revealed -SH groups by such processes, possibly to H_2S (Hutton and Patton 1952).

In experiments utilizing the nitroprusside test as an indicator of sulfhydryl groups, Patton and Josephson (1949) demonstrated that these groups disappear in skimmilk if heated for longer periods of time. On the other hand, the -SH substances did not disappear during prolonged heating of whey. The same conditions were obtained when exhaustively dialyzed skimmilk was used. When lactose was added to the latter a disappearance of -SH substances was re-established. The authors concluded that under the influence of heat the -SH groups disappear by reaction with substances consisting of lactose and casein. In addition, they proved that heat

denaturated whey proteins were being associated physically and/or chemically with casein. Furthermore, the differences in curd characteristics of heated and unheated milk they ascribed in part to this association. An experiment by Sullivan et al. (1957) using serum proteins labelled with S^{35} indicated that substantial quantities of denaturated whey proteins were removed with the casein when heated milk was centrifuged at high speeds.

Data obtained by Boyd and Gould (1957) showed that momentary heating of milk at any given temperature above the critical temperature, produced the greatest quantity of TDS reducing compounds. A portion of these was comparatively stable to decomposition and did not appear to be the precursor of the H_2S. When cream and buttermilk were subjected to the same treatment a larger quantity of H_2S was decomposed when compared with milk and whey. Buttermilk yielded 38 per cent more hydrogen sulfide than whole milk, 103 per cent more than whey and 11 per cent more than cream. These results show that the buttermilk source was more readily activated by heat treatment.

3. Effects on casein and lactoglobulin

Milk is one of the few foods which can be separated into its components, whose properties and behaviour can be studied and independently measured, and then evaluated by recombining them into a reconstituted milk of a previous composition.

Descriptions of chemical changes within the protein phase

of milk indicated that ionizing irradiations could cause some denaturation which could be demonstrated by changes in coagulation time. An increase of rennet coagulation time had been found and, as verified by Wertheim et al. (1957), the coagulation time increased with an increase in dose. However, the degree of change did not seem to be greater than that caused by heat treatment.

As stated by Kraybill et al. (1960) gamma or cathode ray irradiation of milk proteins results in complex changes and it involves not only denaturation but also polymerization degradation and/or molecular rearrangements. These authors determined that about 5.6 Mrad of gamma irradiation was required to induce a molecular alteration sufficient to decrease markedly the biological response to the treated protein. In investigating the mean lethal shocking dose for milk sensitized guinea pigs it was also demonstrated that molecular configurations were responsible for differences in antigenic properties of milk proteins though they might have the same or similar chemical compositions. Further light was shed on changes taking place during the irradiation of milk protein from the viscosity data. As found by the same authors there is a gradual increase in the viscosity of raw skimmilk from 1.498 to 1.574 centipoises with increasing dosage up to 5.58 Mrad. Between 5.58 and 9.30 Mrad the viscosity changed markedly from 1.574 to 1.715 centipoises. At a 9.30 Mrad dose gelation occurred. Hence, it was considered possible that these abrupt changes might reflect an increasing molecular size particularly between 5.58 and 9.30 Mrad.

This polymerization could in turn explain the dramatic decrease in biological response observed with milk sensitized guinea pigs.

The electrophoretic patterns obtained with the casein and whey fractions from gamma irradiated skimmilk provided a further support for the above conclusions. At irradiation doses of 2.79 Mrad and below the only apparent change was a decrease in the casein, albumin and globulin fractions. The residue, however, continued to move in the electrophoretic field as observed in the non-irradiated control sample. At 5.58 Mrad both the α-casein and immune globulin had totally disappeared, and an immobile protein component appeared in both the casein and whey fractions which had the effect of decreasing the mobility of the remaining α-and β-casein as well as that of the α-lactoalbumin and β-lactoglobulin. At a dose of 9.30 Mrad only the immobile components were observed in either the casein or whey fractions. These electrophoretic data suggested that a degradation of protein could occur below 5.58 Mrad and a polymerization of both whey and casein proteins could occur above 5.58 Mrad. In comparison to gamma irradiation, as suggested from electrophoretic data, ultraviolet irradiation induced only a small degree of polymerization in the casein fraction with no polymerization of the whey proteins.

The viscosity increase and decrease in electrophoretic mobilities was finally correlated with the content of -SH. By the applying of a sensitive histochemical-chromatographic procedure an increase in both -SH and -S-S- contents were observed. The change of

these groups in the gamma irradiated samples was marked at 5.58 Mrad and above, whereas, in the ultraviolet treated samples the sulfhydryl content was linearly related to exposure time while there was no indication of any change in the disulfide content. From these data Kraybill et al. (1960) concluded that ultraviolet irradiation and low levels of gamma irradiation induced a denaturation and/or destruction of milk protein with concominant increase of sulfhydryl linkages and alteration in antigenic properties of the protein. High levels of gamma irradiation probably induced a subsequent agglomeration of the protein fragments. The new protein entity had different physical and antigenic properties. The agglomeration was accompanied by an increase in -SH and disulfide bonds, suggesting that the formation of disulfide bridges could be partly involved in the formation of a new entity.

A low temperature irradiation study by Scanlan and Lindsay (1968) produced milk samples with extremely bitter flavor. The pasteurized whole and skimmilk in the frozen state at -80° and $-185^{\circ}C$ were irradiated with a Co^{60} source to 4.5 Mrad. From the similarity of responses of whole and skimmilk they concluded that the fat content did not contribute to that bitter component. The color of the frozen irradiated milk was normal with only a slight churning or destabilization of proteins. When such samples were fractionated by centrifugation and precipitated into fat casein, heat coagulated protein, and serum by acid and heat, the bitter components remained in the serum fraction. Since the bitter components were not dialysable it

was concluded that they were proteins or non-dialysable protein fragments. When these were fractionated on a dextran gel column at least four fractions were collected. However, the bitter flavor was associated with only one fraction which had a ninhydrin and ultraviolet absorption spectrum typical of a protein. By a flavor panellist, regardless of whether the protein was isolated from whole or skimmilk, the flavor was evaluated as extremely bitter, putrid and "wet dog".

Results relating to the influence of ionizing irradiation on purified casein were reported by McArdle and Desrosier (1955). A 2 per cent aqueous solution of the protein was irradiated with cathode rays and the molecular changes occurring were followed by viscosity, -SH, and electrophoretic mobility measurements.

The results for the relative viscosity of casein have shown a slight decrease in viscosity at an irradiation dose of 0.5 Mrep. As the dose was increased the viscosity also increased. At doses in the range of 1 to 1.5 Mrep the increases in the relative viscosity were represented by a straight line. Marked changes were observed when casein was irradiated in the presence of ascorbic acid. The change in viscosity followed the same pattern as that obtained for unprotected casein but the rate of viscosity increase was considerably decreased. The free boundary electrophoretic studies were conduced by a Tiselius apparatus. For the commercial casein two components were apparent, being designated as α- and β-casein. After exposure to ionizing irradiation the pattern of

casein appeared only as a single component, which migrated at a rate much slower than either of the two initial components. When casein was irradiated in the presence of ascorbic acid the pattern resembled that of the control, but the rate of migration for the two components was reduced.

An enzymatic approach with trypsin confirmed also that there is a molecular rearrangement in casein induced by irradiation. By following the reaction rate on casein it was observed that the irradiation by cathode rays altered the protein molecules in such a way that they were more easily hydrolyzed by this enzyme. From these experiments McArdle and Desrosier (1955) concluded that at a dose of about 0.5 Mrep splitting occurred in some of the casein molecules. This was the explanation for an observed decrease in relative viscosity. The molecular splitting was followed by polymerization of fractions which resulted in an increase of viscosity. Polymerization progressed with increasing doses of irradiation until visible coagulation of casein occurred. This point as they stated was observed at doses slightly above 1.5 Mrep. At even higher doses the viscosity could no longer be measured due to casein coagulation. Of interest is that the denaturation pattern outlined here had been observed for the casein during a heat denaturation process (Neurath and Bailey 1954). With increased heat treatment the protein molecules also split, polymerize and coagulate. An increased enzyme reaction rate has also been observed following thermal rupture of the bonds causing an opening of the

molecules. From this point of view cathode ray irradiation was only simulating the known effects of heat treatment of casein.

The involvement of sulfur linkages in solubility changes of protein exposed to large doses of irradiation has been frequently suggested. As assumed by Koch (1958) the polymerization could result from disulfide interchange involving even a chain reaction initiated by the free radicals produced by ionizing irradiation. In this manner hydroxyl radicals from water could react on a disulfide to produce a sulfenic acid and a sulfide radical. The latter could then replace a second sulfide radical in an existing disulfide molecule. Further reaction of sulfenic acid could lead also to a polymerization by a dismutation reaction yielding sulfide and sulfinic acid. Consequently, these possibilities were studied on a model system consisting of a mixture of bis-2,4-dinitro-phenyl-L-cystine and L-cystine. The results have shown that even with large doses no detectable interchange occurred in dry or even aqueous model systems. Hence, the disulfide interchange, as suggested by Koch, could not be considered as the primary cause of casein denaturation by ionizing radiation.

Comparisons of some direct and indirect effects of ionizing irradiation on protein revealed their similarities and differences. The indirect effect was that obtained in dilute solution when precipitation occurred. Using serum albumins as model proteins Alexander et al. (1956) found a marked rise in the protein average molecular weight upon irradiation in dilute solution. As confirmed by them

the increase in weight was due to the formation of a polydisperse range of aggregates which, as revealed by ultracentrifugation and electron microscopy, were in range from dimers up to units of a few hundred molecules. The apparent increase of ultraviolet absorption at 280 nm after irradiation of protein was also confirmed. The increase was explained as due to loss of light by increased scattering caused by large aggregates formed on irradiation rather than due to a preferential attack on the tyrosine residuents which were responsible for this absorption band. The direct effects were those observed by irradiation of a solid state model system. These differed significantly from the previous one by being markedly influenced by the presence of oxygen. In addition the direct effect brought about a true change in ultraviolet absorption which indicated that the tyrosine was really attacked. The extent of this change was much greater than could be accounted for by energy taken up in other amino acid residuals, contributed to the attack on the tyrosine. Irradiation of protein in solid state in the presence of oxygen and in vacuum showed that irradiation in oxygen introduced into protein unstable peroxides, which were capable of initiating polymerization of vinyl compounds. Vacuum irradiated protein was ineffective in such initiation of polymerization.

When aggregates are formed by protein-protein cross linkage or by denaturation of native molecules, it would be expected that the amino acid composition of insoluble aggregate would be similar to that of native protein. In addition, if scission

products are obtained by random cleavage at sites of the most irradiation sensitive amino acids into polypeptide chains, then even these scission products would have an amino acid pattern similar to that of the native protein. In experiments by Kumta and Tappel (1961) 0.1 per cent aqueous solution of proteins were anaerobically exposed to a range of 0.10 - 50 Mrad of gamma irradiation. Insoluble aggregates were separated by centrifugation and ultrafiltration. Soluble proteins were precipitated by trichloroacetic acid (TCA) and thus separated from scission products that were present. When these three fractions were analysed for amino acid composition it was found that cystine, methionine, histidine, phenylalanine, tyrosine, threonine, and serine were most susceptible to irradiation damage. The lability rank, however, varied with the protein. These amino acids were degraded even at doses lower than 0.1 Mrad in contrast to other acids such as aspartic and glutamic acids, alanine and lysine, which were among the most stable. Furthermore, the amino acid pattern of the scission products and insoluble aggregates was different from that of native protein or TCA precipitable protein, at a given level of dose. These findings strongly suggested that protein denaturation or polymerization was not the only cause of the formation of insoluble aggregates and, moreover, that a simple hydrolysis induced by irradiation was not the mechanism of protein fragmentation.

4. Mechanism of action of ionizing irradiation

The irradiation effects reported up to now have been assessed by measurement of some physical parameters, such as, viscosity, electrophoretic mobility, and solubility, or by measurement of biological activity. However, such assessments gave little information about the initial alterations and the mechanisms in general, which could take place in protein molecules after the absorption of irradiation doses. In recent years the electron spin resonance (ESR) technique has become available which appears to be well suited for better understanding of these problems. In most organic molecules, such as amino acids, lipid, protein, and nucleic acid, two electrons, according to the Pauli principle, share an orbit with their spin aligned in a non-parallel alignment. Strong ionizing doses of gamma, cathode or X-rays can eject electrons from these orbits. If a molecule loses a single electron the resulting molecule, called a radical, will have a single unpaired electron. This electron is now free to flip over in an externally applied magnetic field. If the spin of an odd electron of a radical were completely free from perturbing influence of its environment, surrounding hydrogen atoms, amino, methylene, methyl, etc., groups, its resonance would be a single sharp line. Fortunately, the electron resonance signals are often rich with information about the environment of the odd electron which usually indicates its true location. Most instruments used for the detection of ESR, plot the intensity of absorption at a fixed frequency (9000 Mc/s) as a function of a changing magnetic field

(3000 ± 100 gauss).

As found by Patten and Gordy (1961) by ESR different free radicals were obtained by irradiation of differing amino acids, but generally only one free radical was formed and stabilized within a given amino acid. The signal reported for powdered glycine irradiated and observed at room temperature was a triplet. When irradiated and observed at a temperature of liquid nitrogen ($77^{o}K$) a doublet resonance was observed. The free radicals obtained by irradiation of powdered alanine at room temperature gave a quintet, while at $77^{o}K$ a triplet ESR pattern was obtained which probably arose from the free radical of the form: $CH_3 - \dot{C}H - COOH$. When irradiated at room temperature D,L-valine gave an ESR pattern rich in hyperfine components spread over some 180 gauss, and when irradiated and observed at $77^{o}K$ a single line was obtained spread over only 55 gauss. The free radical produced at low temperature might have a structure in which the electrons spin would be shared equally between the two oxygen atoms of the carboxyl group through a switching of the double bond. The interaction with CH and NH_2 groups would then broaden the resonance. L-leucine, when irradiated and observed at $77^{o}K$, gave an ESR pattern which appeared to be a superposition of two different free radicals. One of them, a sharp doublet of 40 gauss spacing, could arise from a negatively charged radical of the form: $R\text{-}CHNH_2\text{-}C\begin{matrix}O^- \\ OH\end{matrix}$. Cystine and cysteine have similar ESR patterns when irradiated at room temperature. The common free radical was found to be $HOOC\text{-}CHNH_2\text{-}CH_2S$. e.g. the electron spin is concentrated mainly in an orbital of the

sulfur atom. When the two amino acids were irradiated and observed at 77°K the spectra differed, but the unlike patterns were transformed into the similar one when warmed to room temperature. When methionine was irradiated the resonance obtained suggested a free radical in which the electron was concentrated on a hydrocarbon group. But, there was, however, a weak cysteine-like component which became evident when the sample was recooled to 77°K.

From all these ESR patterns Patten and Gordy (1961) concluded that at a low temperature there is frequently evidence for more than one kind of the free radical in an amino acid than at room temperature. The characteristic differences which are dependant on low and room temperature irradiation would seem to result from the greater rigidity of the structure at low temperature, which would prevent both the escape of odd electrons, and the reorientation or rearrangement of the parts of damaged molecules to form the most stable free radical. The ESR pattern at room temperature revealed by a hyperfine structure of symmetry would be considered not as a primary free radical but as a secondary one produced by molecular motions after the primary event of ionization. In agreement with these suppositions was the finding that the sample irradiated at the lower temperature and subsequently allowed to warm to room temperature gave an ESR pattern that was recorded directly when the sample was irradiated at room temperature.

When an ESR study was conducted with proteins instead of

individual amino acids, the ESR patterns obtained at room temperature were mainly of two types. One of these was a doublet similar to that of glycine, the other a pattern specific for irradiated cystine. Hence, casein with glycine content of 1.8 per cent and cystine and cysteine 0.3 and 0 per cent, respectively, gave at room temperature a resonance pattern of a weak doublet. A similar pattern was registered for collagen which contained 23 per cent glycine and no cystine or cysteine (Patten and Gordy 1964). On the other hand, serum albumin rich in cystine and cysteine and low in glycine content had a resonance pattern typical of cysteine superimposed with a weak glycine doublet. When the ESR study was conducted with protein at a temperature of liquid nitrogen the ESR registered was completely different from those at room temperature. But, as the irradiated samples were warmed to room temperature the electron spin migrated to the sulfur of the cysteine or cystine constituents or to the glycine, and the ESR pattern acquired was the same as obtained by direct irradiation at room temperature. Such a transfer of the electron was quantitative in proteins which had about 10 per cent of sulfur-containing amino acids. In the same proteins, when exposed to air, the doublet resonance started to decay. In some proteins the doublet was converted to a singlet, which in turn decayed further. These effects clearly reflected the interaction of oxygen with the free radicals.

Casein, as the major milk protein had a particular importance in the ESR study. Moreover it was of interest as a protein because it contains only a small amount of glycine and no meaningful

amounts of cysteine or cystine. As mentioned earlier, casein resonance was principally a doublet which was not so well resolved as that for collagen. In addition to this doublet there were weak satellites corresponding to the spin density on the alanine, glycine, leucine and glutamic acid residues. In contrast to this there was no evidence of the cystine-like resonance, which includes also that of methionine, although methionine contributes with 2.5 per cent in weight of this protein. This suggested that -R $CH_2S\cdot$ radical was not readily formed at the methionine centres. On exposure of the sample to the air the casein doublet decayed very slowly, without change of form, indicating that this protein radical was rather inert and consequently not easily transformed to a peroxide type of molecule.

Similar to casein many other proteins in dry state, irradiated at room temperature, did not reveal an ESR composite spectra of constituent amino acid, e.g., they contained the spectra of the sulfur pattern and doublet of the glycine radical. In addition, the energy absorption forming the primary radical was followed by a migration of odd electrons leading to the formation of the secondary radical. Two principally different hypotheses have been advanced to explain the above findings. Gordy et al. (1955) proposed an intramolecular migration mechanism by which electrons or electron holes were assumed to migrate along the peptide helix until they reach the cystine or glycine groups which served as trapping sites. The alternative one was an intermolecular migration mechanism. Hydrogen

atoms, small molecular fragments, or single electrons, formed by ionization, by homolytic bound rupture were assumed to diffuse through the solid state protein and interact with neighboring molecules to form the secondary radical.

As found recently by Copeland et al. (1968) in experiments using adenine as a radical scavenger, the secondary sulfur and doublet type radicals were formed primarily by an intermolecular mechanism.

The intermediates in the intermolecular formation of secondary protein radicals are known. Since heat treatment was usually required the mechanism could involve a rearrangement of the primary radical with the liberation of diffusible radical intermediates. As had been suggested by Braams (1963) it is likely that H· atoms could play the role of intermediates in such reactions. In addition, a transient formation of HO_2 radical, probably formed by decomposition of organic peroxide radicals at the initial site of odd electrons, could also play the role of the diffusible intermediate.

5. The effect of additives on off-flavor development

Various methods have been tested and found useful in preventing or decreasing the off-flavor development and other physicochemical changes of protein constituents induced by irradiation of milk and milk products. Basically, they consisted of lowering the temperature during irradiation, avoiding the oxygen by using

vacuum or by irradiation in an atmosphere of nitrogen.

The early experiments by Proctor et al. (1952) revealed the potential of the use of vitamin C (ascorbic acid) as a free radical acceptor (FRA). The use of this additive received the support of the United States Food and Drug Administration since vitamin C was considered harmless and was on the list of permissible food additives. In prevention of off-flavor development the following will serve as a general illustration:

$$\text{Flavor} + \cdot OH = \text{Flavor}_{ox} + OH^-$$

$$\text{Flavor} + \cdot OH + \text{FRA} = \text{Flavor} + \text{FRA}_{ox}$$

The experiments reported by McArdle and Desrosier (1955) established the value of vitamin C as an additive preventing irradiation flavor in milk. At that time the protective effect of vitamin C was explained as the result of a partial uptake of the irradiation energy by ascorbic acid. Soon this assumption was replaced by a hypothesis that vitamin C could play the role in the milk as a free radical acceptor. If this assumption is valid, then vitamin C should have a mobile diffusible constituent with an odd electron, which in turn would leave behind the vitamin residue also in a form of a free radical. For such assumption the proof was provided later in an autoxidation study of the ascorbic acid by Lagercrantz (1964). As found by this author, 0.1 M ascorbic acid dissolved in 0.1 N sodium hydroxide in presence of air oxygen gave a free radical doublet structure in an ESR measurement. The free radicals were found to be present between pH 6.6 and 9.6.

The hyperfine structure of the ascorbic acid revealed a triplet splitting of each of the two lines of the doublet. This ESR pattern suggested that the unpaired electron is located in position 2 and that a coupling existed between the electron and the proton located on the carbon atom 5. The triplet splitting was explained as due to further interaction of odd electron with two equivalent protons attached to the carbon atom in position 6. Very likely due to steric reasons no other coupling could be observed.

The existence of ascorbic acid free radicals in irradiated milk was not confirmed directly. If it existed in milk it would be still unclear whether it was produced directly by irradiation induced homolytic cleavage at HO-groups or by secondary process initiated by ·OH and/or $HO_2^{\cdot}$ radicals obtained from the radiolysis of the inherent moisture.

In a parallel study (Proctor and O'Meara 1951) it was confirmed that in dilute solution ascorbic acid was more sensitive to irradiation than the concentrated solution. Moreover, the loss of ascorbic acid on irradiation was less "in situ" than in pure ascorbic acid solution. Since chemical effects induced in dilute aqueous solution were apparently indirect, by the interaction of free radicals produced by radiolysis of water, the above findings could be expected. Such an apparent interaction was later even confirmed (Barr and King 1956). Since the radiolysis of water

proceeds as follows:

$$H_2O = H\cdot + \cdot OH$$

$$H\cdot + O_2 = HOO\cdot$$

$$e^- + H_2O = H_2O^-$$

$$e^- + H^+ = H$$

$$H_2O^+ = H^+ + OH\cdot$$

or in more detail: $H_2O = H_2O^+ + e^-$

$$H_2O^- = OH^- + H$$

$$OH^- = OH\cdot + e^-$$

the interaction could involve:

$$AH_2 + \cdot OH = HOH + AH\cdot$$

$$AH_2 + HOO\cdot = H_2O_2 + AH\cdot$$

$$AHO + O_2 = AHOO\cdot$$

$$AHOO\cdot + AH\cdot = 2A + H_2O_2$$

where AH_2 represents the ascorbic acid. As Barr and King (1956) did not find a reaction of the type -

$$AHOO + AH_2 = AH\cdot + A + H_2O$$

the chain utilization of oxygen should be excluded in these interactions, which would be more consistent with the protective effect of ascorbic acid observed during irradiation.

On the other hand, a different mechanism for ascorbic acid free radical interaction was proposed in absence of oxygen (Rao 1962):

$$AH_2 + H\cdot = AH\cdot + H_2$$

$$AH_2 + \cdot OH = AH\cdot + H_2O$$

$$AH\cdot + H_2O_2 = A + H_2O + \cdot OH$$

$$AH\cdot + \cdot AH = A + AH_2$$

As demonstrated by the same author in presence of CO_2 in water the yield of A is very much lower suggesting a direct competition

in solution for the available free radicals. This evidence suggested that oxidation of ascorbic acid could be suppressed only in the case when there is a stronger competitive scavenger present in solution.

A more recent study by Harper et al. (1969) has shown that AH· free radical could be transformed further into other forms of free radicals due to a resonance effect existing at the double bond C_2 and C_3.

For shielding irradiation effects on sodium caseinate Littman et al. (1957) suggested the use of aldehydes as additives. As demonstrated by them the release of hydrogen sulfide due to radical reactions ·H + ·SH in presence of aldehyde has been decreased up to 15 per cent as compared to unprotected solution.

Other compounds were also reported to have a protective role in retarding protein denaturation during irradiation. Henriksen et al. (1963) found that an irradiation induced unpaired electron from protein could be readily transferred to a sulfur compound of a small molecular weight. The data also indicated that at room temperature such a transfer to sulfur took place in the course of minutes, and at a concentration of the order of 30 per cent thiol the transfer was practically complete. Such an intermolecular transfer presumably required a collision between the molecules exchanging spins. One possibility as suggested by the authors would be a hydrogen transfer:

$$\text{Protein}\cdot + \text{RSH} = \text{Protein H} + \text{RS}\cdot$$

This mechanism which actually constitutes a repair mechanism would require the spins to be induced on the surface of the protein. The second possibility would be that the hydrogen atom or a small radical fragment was abstracted from protein to react subsequently with thiol

$$A\cdot + RSH = AH + RS$$

which transfer would then form a modified protein.

The above results were valid for dry systems of protein and thiol mixtures. If the system were in an aqueous solution there would be a slight chance for such a spin transfer. Moreover, as confirmed by Barron and Flood (1950) the thiols such as 2,3-dimercaptopropanol (BAL) and propane 1,3-dithiol are readily oxidized to disulfides by the free radicals formed only from the radiolysis of water. Disulfides as indicated by Henriksen et al. (1963) are even in dry systems poor acceptors for unpaired spins. Nevertheless, the readiness with which the thiol group recombines with free radicals from water would indirectly provide a protection to the labile site of the protein molecule present in the same solution. As confirmed by Kumta et al. (1962) all the irradiation labile amino acids of a protein in the presence of thiols received the protection to the same extent. This finding would favor the view that the thiols were operative on a basis of a free radical scavenger mechanism at least for those radicals obtained from radiolysis of water.

C. Determination of Sulfydryl Groups in Skimmilk Powder

1. Amperometric methods

A widely accepted and perhaps the most widely used technique for the determination of -SH groups in proteins has been that of amperometric titration.

When an -SH group bearing protein is titrated with aqueous silver nitrate at a platinum electrode against a suitable reference electrode, the insoluble very slightly dissociated silver mercaptide is precipitated and a negligible current flows until there is an excess of silver ions in solution. At this point the diffusion current rises sharply and the end point is then obtained graphically by plotting current readings against the volume of standard silver nitrate added and locating the point of intersection of the two straight lines.

Early application of amperometric titration (Bloksma 1959) used a stationary Pt electrode along with stirring of the substrate in electrolyte. It was also demonstrated that 0.001 M silver nitrate was preferable as a titrant to mercuric nitrate. Using a hook-type rotating Pt electrode, 600 rpm, Sokol et al. (1959) showed this type of an electrode to be superior to the stationary one since fouling could be tolerated. The electrolyte was made up of tris buffer at pH 7.5. They also established that higher volumes of -SH groups could be obtained by their method than were obtained by the method of Bloksma. They also claimed that the dispersion and titration had to be carried out at 2^{o}

to prevent -SH loss and that the addition of EDTA would minimize the effect of interfering metal ions. Urea from 6 to 8 M was used to unwrap proteins and to unmask the presence of -SH groups.

Matsumoto and Hlynka (1959) following Bloksma's method designed an airtight vessel for the titration as it was assumed and partly confirmed that the presence of oxygen influenced the -SH group determination. Mecham and Knapp (1966) and Tsen and Bushuk (1968) were making use of nitrogen flushing in the titration cell and had extended estimations to include -SS- determinations after treatment with sulphite followed by 6 M urea and titration with silver nitrate.

By using a rotating Pt electrode at a speed of 150 rpm Hutton and Patton (1952) have shown that the sources of -SH groups and volatile sulfides in milk are those of serum proteins, particularly the albumin, and of that protein which is associated with the fat globule membrane. Results for raw skimmilk, casein and whey in an electrolyte consisting of 50 per cent alcoholic ammonium nitrate-ammonium hydroxide were 0.117,0.000 and 0.127 meq/1 of -SH group expressed as cysteine. These data indicated that the whey protein is the source of -SH groups and that casein is devoided of them. Fractionation of the serum protein into a number of components by ammonium sulfate additions and pH adjustments revealed that β-lactoglobulin is the source of practically all the -SH groups present in whey. As shown by the same authors, the silver nitrate titration, when conducted in aqueous medium, appeared to measure

the same quantity of -SH groups in heated skimmilk as the nitroprusside and thiamin disulfide methods. Higher results were obtained when an alcoholic medium was used. It seems probable, therefore, that in alcoholic mediums the total number of -SH groups in milk, the free and masked ones, were determined.

Slightly less than one half of the -SH groups detectable in skimmilk by ortho-iodosobenzoate method were titrated using the argentometric-amperometric titration. Similar results have been found in the titration of crystalline β-lactoglobulin which has been reported to contain from 1.11 to 1.30 per cent cysteine using ortho-iodosobenzoate method, while 0.53 per cent was obtained using argentometric-amperometric procedure.

Zweig and Block (1953) also applied argentometric-amperometric titration in their study of the effect of heat treatment on the sulfhydryl groups in skimmilk and nonfat dry milk. Instead of alcoholic ammoniacal solution, 0.1 M sodium acetate was used as a titrating medium. In this case, in contrast to cysteine, the milk protein titrations showed a sharp increase in diffusion current prior to the end point followed by a slow drift towards a zero diffusion current. This phenomenon continued in sodium acetate medium until the end point was passed after which the diffusion current increased proportionally to the amount of silver ions in solution. The drift encountered in the case of milk protein, but not in the case of titrations of soluble mercaptans, was explained on the basis that an appreciable time is required for

silver ions to penetrate the protein molecules. Although the authors found that the quantity of -SH groups in milk and its derivatives varies from batch to batch of milk, even when obtained from the same farm, the distribution of these groups in milk, whey and casein gave typical values. In agreement with amperometric titrations in alcoholic ammoniacal solution, the results in sodium acetate solution have shown that all the -SH groups of milk were in the whey proteins. The values calculated as milliequivalents of cysteine per liter of milk were for skimmilk 0.264, raw whey 0.316, and for the protein-free whey and casein 0.000.

In the study of -SH and -SS- groups in milk proteins Yoshino et al. (1962) have demonstrated that some mercaptans like cysteine do not yield theoretical -SH values by the amperometric titration. Titrating cysteine in buffer solutions such as sodium acetate, ammonium nitrate and tris at different pH levels no theoretical value was obtained. Five to fifty per cent error resulted from the excess binding of silver ions as RSAg mercaptide, and to the formation of complexes like $(RSAg)_2Ag^+$. When titrating milk and β-lactoglobulin in sodium acetate buffer the highest values were obtained at pH 10.25. The titratable -SH groups were 0.235 mM/1 of milk as compared to 0.264 mM reported by Zweig and Block (1953). The highest titers in ammonium nitrate and tris buffers were 0.18 and 0.22 mM -SH/1 of milk respectively. The -SH groups in raw milk titrated in ammonium nitrate buffer were higher than the data reported by Hutton and Patton (1952) for titrations made

in 50 per cent alcohol. The -SH content of β-lactoglobulin in sodium acetate at pH 10.25 yielded 1.063 g of cysteine, while titrations in tris and ammonium nitrate buffers yielded only 70.3 and 86.5 per cent, respectively, of the previous value. Tris buffer at pH 7.4 was used by Benesh et al. (1955) in the amperometric titration of -SH groups in crystalline proteins. At a higher pH more -SH groups were titrated. On the other hand Burton (1958) obtained more -SH groups in β-lactoglobulin when it was titrated in tris buffer as the pH was decreased. Contrary to this finding Yoshino et al. (1962) reported fewer -SH groups in cysteine, β-lactoglobulin and milk with this buffer when the pH was lowered. The -SS- groups in milk proteins were also determined amperometrically by the latter authors. The disulfide groups were reduced to -SH with sodium sulphite and urea yielding 1 M -SH as follows:

$$RSSR + SO_3^{2-} \longrightarrow \underset{\text{thiol}}{RS^-} + \underset{\text{S-alkyl thiosulfate}}{RSSO_3^-}$$

Hence, the disulfide content was the difference between the -SH content before and after reduction of -SS- groups. As found by the same authors, the effect of 1 per cent sodium lauryl sulfate (SLS) was more effective than 8 M urea for activating the -SH groups. But a concentration of 8 M urea was approximately twice as effective with sulphite than the SLS when the disulfides had to be reduced. In the same study no -SH groups were found in casein. The cystine content found was 0.265 per cent which equalled 0.300 mM of -SS-.

Sasago et al. (1963) determined the -SH content in milk and β-lactoglobulin by amperometric titration and compared the results with those obtained by the p-chloromercuribenzoate (PCMB)-dithizone(D) method. The -SH values for the latter method were nearly the same as those found in amperometric titration in 8 M urea, and about 30 per cent less than the amperometric values in sodium acetate ammonium hydroxide buffer. After the silver ion had reacted with all of the -SH groups in the amperometric titration PCMB was added to the milk sample gradually. The PCMB replaced silver ion which had been bound, presumably, with -SH groups. The amounts of released silver were different in sodium acetate-ammonium hydroxide buffer, 8 M urea and 1 per cent SLS solutions, but the amounts of PCMB added were nearly constant. The ratio between the released silver ion and the PCMB were 1.5:1 in sodium acetate-ammonium hydroxide buffer, 1:1 in 8 M urea, and 1.4:1 in 1 per cent SLS. Hence, variations in amperometric titration of milk might be explained to the nonspecific binding of metal ions and/or to the binding of the metal to only a portion of the -SH groups.

Beeby (1964) using the dropping mercury electrode titrated K-casein amperometrically in 8 M urea in a borate buffer pH 9 with phenylmercury-acetate after adding sulphite. The titration of 18 different samples of K-casein yielded an -SS- content of twice the value previously reported. These results suggest that the K-casein might contain cysteine rather than cystine. However,

no titre was obtained in the absence of sulphite. Treating the protein solution from the Sephadex G-25 column with EDTA and then removing the EDTA by passing the solution through Sephadex and by adding 0.4 M sodium chloride the presence of titratable -SH groups was also observed. This result indicated that K-casein contained cysteine in a masked form and calcium appeared to be implicated in the masking. The -SH groups could be inaccessible because of an aggregation maintained by calcium. Since sulphite forms an insoluble calcium salt, it could remove calcium and allow disaggregation to occur. However, even when calcium has been removed by 0.5 per cent EDTA or by 1.5 per cent oxalate, the availability of the -SH group is lost quickly suggesting that aggregation through other means, such as hydrophobic bonding, may occur.

The use of an amperometric adaptation of the dead stop titration, which depends on the depolarizing action of iodine on a polarized Pt cathode, was also applied to the determination of -SH groups of milk protein. A pair of bright Pt electrodes with a potential of 10 - 20 mV immersed in the protein solution was used with iodine and/or o-iodosobenzoate as reagents. On the basis of the assumption that the stoichiometry of the oxidation corresponded to the formation of the -SS-, the cysteine contents for casein and crystalline β-lactoglobulin were found to be 0.000 and 1.300 per cent, respectively (Larson and Jenness 1950).

In the analysis of β-lactoglobulin, using o-iodosobenzoate procedure, essentially the same titration values were

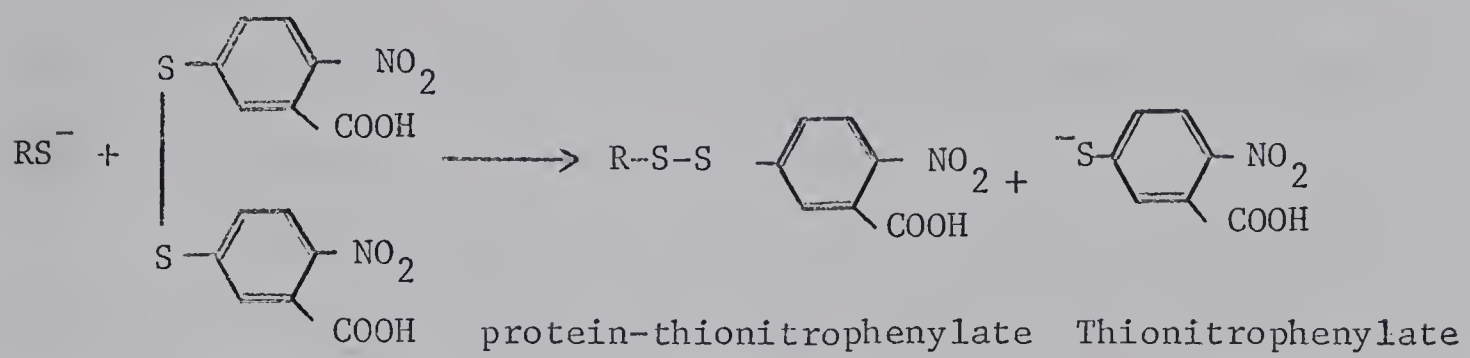
obtained for the quanidine denaturated as for the native protein. A further advantage of the o-iodosobenzoate as a reagent is the fact that the reagent reacts with highly reactive -SH groups and does not carry the oxidation to further stages. Fresh milk proteins apparently do not contain such reactive groups but there is evidence that they are formed during heat treatment of the milk serum proteins (Larson and Jenness 1950).

In casein analysis the end point determination is also done by the starch as indicator instead of amperometry but the results for the content of -SH groups are higher and less reliable. Iodine does not form a visible complex with starch until the iodine normality is 1 to 10 x 10^{-6} N depending somewhat on the type of starch used. However, casein adsorbs part of the iodine from the aqueous phase and hence the blue color with starch appears at higher concentration of added iodine. Hence, when starch is used as an indicator the casein appears to have a considerable reducing capacity, amounting to 0.15 per cent cysteine.

The amperometric adaptation of the dead stop titration was also used for the -SH group determination in whole milk. In this analysis the milk had to be diluted before the acid and iodide were added to avoid precipitate formation which clogged up to the electrodes and interfered with the galvanometer readings of the current, which flows while the cathode is being depolarized by the iodine.

2. Spectrophotometric methods

The Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid), also know as DTNB, has been a useful tool for assays of free sulfydryl groups in many proteins. The reaction of this reagent with -SH groups of protein is presented below:



DTNB — protein-thionitrophenylate complex — Thionitrophenylate anion

The conventional assay for the -SH group in protein is carried out by incubating the protein with DTNB in 0.2 M phosphate buffer at pH 8.0. The quantity of liberated highly colored thionitrophenylate anion is then determined spectrophotometrically at 412 nm (E_{mole} = 13,600)(Ellman 1959). To obtain a yield of one mole of thiophenylate protein and one mole of thiophenylate anion the reaction is usually carried out above pH 7.0.

Lyster (1964) applied this spectrophotometric method to the determination of -SH groups in milk and skimmilk powder. The procedure involved determination of free -SH groups followed by urea treatment to determine the total -SH content. Masked -SH groups were then calculated by the difference. The method also involved the use of the most specific and sensitive organic mercuric compound for -SH groups - PCMB. The milk proteins were first incubated with this compound followed by the addition of DTNB. The color developed in a proportional intensity when -SH

content was in excess of PCMB. The equivalence point was plotted graphically by extrapolation to zero amount of PCMB added.

Sedlak and Lindsay (1968) applied Ellman's reaction using tris buffer and EDTA as the reaction medium in a manner similar to that used for amperometric titration. They were also able to establish with certainty that the DTNB reagent was reduced with -SH groups on a mole to mole basis.

Recently the DTNB reagent has been used for the detection of -SH groups in thin layer plates (Glaser et al. 1970). Sodium borohydride was used to reduce -SS- to thiols which were then determined with DTNB. The detection limits in this work were in the order of 2×10^{-9} moles of cysteine.

Kalab (1970) studied the factors affecting the Ellman determination of -SH groups in skimmilk powder and gels and confirmed that the stability of thionitrophenylate is increased over the pH range of 7 to 9 when the solutions were kept at 4^{o}. However, at room temperature a rapid decline of the color of the anion was observed. These results also showed that the amount of detectable free -SH groups decreased rapidly after the skimmilk powder had been dissolved. It decreased to 90.1 per cent of the initial value after 10 and 86.4 per cent after 20 min, 77.9 after 1 and 59 and 33 per cent of the initial amount after 2 and 4 hrs, respectively. As expected the minimum stability was observed at pH 7 whereas, high stability was obtained at pH 9.5 to 10.0. The highest free -SH concentrations were obtained with

milk powders dissolved in water which already contained DTNB. From these results it was suggested that the direct Ellman procedure might have a serious disadvantage, presumably due to the tendency for the liberated thionitrophenylate to autoxidise, particularly when the incubations were prolonged. Contrary to expectation the bubbling of nitrogen gas through milk samples before and after addition of DTNB did not affect the stability of the thionitrophenylate in solution. However, the stability increased in the presence of ascorbic acid. Nevertheless, this protective property of ascorbic acid could not be used, since this compound is also susceptible to oxidation in alkaline medium forming brown oxidation products which interfere with the spectrophotometric assay of -SH groups.

Kalab (1970) also determined that maximum exposure of the masked -SH groups of skimmilk powder occurred with 3 M guanidine hydrochloride, but a greater absorbance stability was achieved in 5 M urea. The unmasking effect of both compounds was instantaneous. SLS in 1 per cent concentration acted slowly in exposing masked -SH groups. In presence of this detergent the rate of the reaction between DTNB and liberated -SH groups reached its maximum 60 min after the milk had been mixed with the SLS solution.

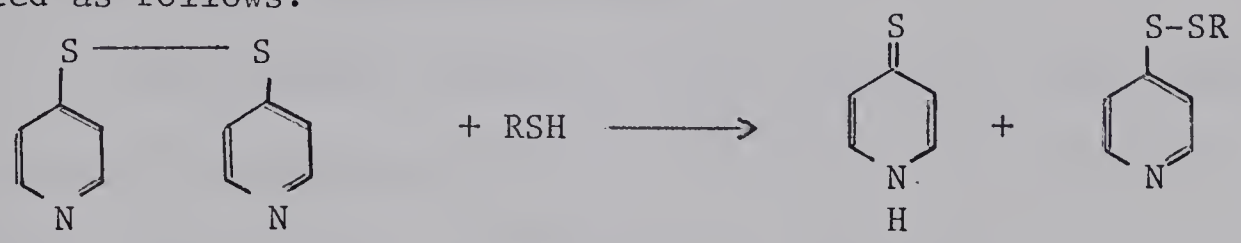
Milk -SS- groups were also determined with DTNB after their reduction to -SH groups. Since DTNB reagent contains an -SS- linkage and is subject to the same reducing reactions as the protein under study, the excess reducing agent must be

destroyed or removed before the addition of Ellman's reagent by a procedure that does not affect the -SH value. As proposed by Brown (1960) sodium borohydride ($NaBH_4$) was accepted because it could be destroyed by acidification or the addition of acetone. An experimental procedure recommended by Kalab (1970) involved 3 N hydrochloric acid:acetone 1:1(v/v) which resulted in color stable for more than 40 min at room temperature. By using this procedure Kalab obtained from 1 g of skimmilk powder 0.425 µM of free -SH and 0.98 µM of total -SH, and after reduction with $NaBH_4$ in 5 M urea, the total S both in -SH and -SS- groups was 9.0 µM.

A modification of the Ellman procedure for the determination of -SH groups of protein, which involved the isolation of thiophenylated protein, was reported by Butterworth et al. (1967). With this modification -SH analysis has been achieved on -SH containing protein in the presence of a reduced thiol such as dithiothreitol(DTT) on insoluble proteins or on a protein rendered insoluble by denaturation. In the application of this method an excess of DTNB was incubated at room temperature. The reaction mixture was then applied quantitatively to a molecular sieving column, Sephadex G-25,50 or 100, and the TNB-protein eluted was collected with good separation from the excess of DTNB free thionitrophenylate anion and small molecular weight mercaptan-thiophenyldisulfides. Then to the TNB-protein an excess of DTT was added to cleave the mixed -SS- bond. The amount of thionitro-

phenylate anion was assayed spectrophotometrically and then reported as moles per mole of protein applied to the column or to an aliquot of the protein in effluent collected.

The determination of -SH groups by -SS- exchange with ortho-and para dithiopyridines has been described by Grassetti and Murray (1967). The corresponding pyridones formed are illustrated as follows:



4, 4'-dithiodipyridine (4-PDS) 4-thiopyridone (4-TP)

The UV spectra of the pyridones were found to be quite different from the parent disulfides and with simple thiols and colorless proteins the reaction was readily followed spectrophotometrically. The sensitivity of the method was not influenced by background absorbance due to 4-PDS (324 nm), since the disulfides exhibit no absorption at the peak wavelengths of their corresponding pyridones (307 nm). In comparison with Ellman's reagent the use of 4-PDS reduces the background interference almost tenfold and provides a means for direct spectrophotometric analysis in determining the kinetics, as well as the number of reactive -SH groups in protein (Ampulski et al. 1969).

The presence of -SH groups in freshly prepared K-casein as found by Beeby (1964) was for many years a subject of controversy. But contrary to his procedure the other authors did not

use strong disaggregating conditions in the analysis of casein. Hill (1964) working on whole casein and using an even more drastic method for its disaggregation, such as proteolysis with Pronase enzyme, observed only the presence of cysteine. But no indication was obtained that the enzyme affected the -SS- bonds in any way since it is well known that -SH -SS- exchange reactions could occur during such limited proteolysis.

More recently Wallace and Aiyar (1969) for three different casein preparations, a) from fresh milk, b) from skimmilk separated industrially, and c) industrial acid casein found the presence of both cysteine and cystine in a proportion of 1:1 by weight and in the molecular ratio of 2:1. Their method was essentially a drastic acid hydrolysis of casein with estimation of -SH and -SS- in the hydrolysate. The hydrolysis was carried out under reflux with a 1:1 v/v mixture of 98 per cent formic acid and concentrated hydrochloric acid for 7 hrs at 105°. As claimed by the authors, the presence of formic acid affected a protection against metal catalysed oxidative destruction of cysteine. During the digestion oxygen free nitrogen was bubbled through the digest. These conditions of hydrolysis shortened the digestion time and thus avoided some destruction of cystine and cysteine. Then the hydrolysate was adjusted with alkaline to pH 3.7-4.0 which allowed the precipitation of the humin material and its removal by filtration. The clear filtrate obtained was then used for the determination of cysteine and cystine using nynhydrin as reagent. The

pink color developed at 100° was determined spectrophotometrically at 575 nm. Determination of the cystine was done in a separate aliquot which was adjusted to pH 5.0 reduced by an excess of sodium sulphite and the color developed as indicated above. Since different casein preparations gave similar results Wallace and Aiyar (1969) concluded that ordinary processing does affect the -SH and -SS- balance of whole casein and, moreover, that the -SH group might not be as labile as is commonly believed.

As already indicated, the organic mercurials appear to be most specific to react stoichiometrically with protein -SH groups. The amount of reagent bound can be measured either indirectly from the excess of unreacted reagent or directly from an increase in absorption at 255 nm which was assumed to be derived solely from protein mercaptide complex. As found by Boyer (1954) the reaction of PCMB with β-lactoglobulin at pH 4.6 in acetate buffer follows the course of a second order reaction. The relative increase at the absorption maximum at 230-235 nm accompanying mercaptide formation is small compared to the increase in 255 and 250 nm which amounted to 213 and 164 per cent, respectively. According to Boyer it was fortunate in the application of this procedure that the maximum absorption changes associated with mercaptide formation occurred in the region where there is a minimum in the UV absorption of proteins. It was also found that some of the -SH groups present reacted much more rapidly at pH 4.6 than at pH 7.0. The factors which determined the reactivity of -SH

groups have not been established although steric effects and hydrogen bonding could be considered as an explanation.

A further development of the above method has been reported by Suzuki et al. (1969). After interaction with the organic mercurial p-hydroxymercuribenzoate the excess of unbound reagent was removed by dialysis or gel filtration on a column of Sephadex G-25 and the amount of mercury bound to -SH groups of the protein sample was measured directly by atomic absorption spectroscopy. The estimation was performed on micromolar amounts and due to interference caused by the absorption of air, instead of the resonance line for mercury at 1849 Å the second resonance line at 2537 Å was used. The application of mercuric chloride instead of p-mercuribenzoate resulted in a small but significant decrease in the recovery of the number of -SH groups.

As stated by Suzuki et al. (1969) the protein -S-Hg-Cl mercaptide complex may have a smaller binding constant than that of a protein -S-Hg-R mercaptide, where R = p-hydroxy mercuribenzoate. It is also possible that a mole of mercuric chloride may sometimes bind two -SH groups, whereas, the organic mercurial does not. Finally, the increased sensitivity provided by the atomic absorption technique is remarkable in that a 30-fold decrease in the amount of protein required for analysis by the usual spectrophotometric method is possible.

The scope and limitations of the large numbers of methods for the determination of thiol groups in protein in general have

been evaluated. The methods applied in milk protein analysis are also numerous but they were not assessed separately. Nevertheless, the conclusion may be the same for both groups as no single method has emerged which might be superior to all others.

III. MATERIALS AND METHODS

A. Source and Preparation of Skimmilk Powder

Two sources of skimmilk powder were used in this investigation, (a) commercially dried high heat or "Baker's" grade powder manufactured by a local firm and (b) laboratory dried powder. The laboratory dried skimmilk powder was made from freshly separated bulked milk. The milk fat of the skimmilk before drying was in the range of 0.06 to 0.07 per cent determined by the butyl alcohol method. Before drying, the milk was heated in a laboratory batch pasteurizer and colled to temperature below 10^{o}C.

In the experiment with skimmilk powder containing ascorbic acid or ascorbyl palmitate as additives, each batch of skimmilk before drying was divided into 9 portions, a control without additives and portions containing 0.05, 0.1, 0.2 and 0.4 per cent ascorbic acid or 0.1, 0.2, 0.4 and 0.8 per cent ascorbyl palmitate.

As small volumes of milk were involved in this investigation they were dried in a Virtis Model 142 laboratory freeze drier. After completion of drying the vacuum was released with nitrogen gas. The dried milk was then packed in cans with clenched covers in a vacuum oven using a vacuum of 100 mm Hg which was subsequently released again with nitrogen gas. The cans were then sealed and stored at 4^{o} until used.

In addition to the skimmilk powder preparations described above, powder containing radioactive ascorbic acid was prepared. This powder was prepared by adding 5 ml of an aqueous solution of 0.3 mg of C^{14}- ascorbic acid (activity 1 x 10^{-4}mC, New England Nuclear, Boston, USA) to a 3 g sample of laboratory dried skimmilk powder containing 1 per cent of unlabelled ascorbic acid as carrier. The slurry so obtained was mixed in a closed test tube using a vortex mixer. After drying in a vacuum oven it was packed as described above.

B. Irradiation of Skimmilk Powder

1. Irradiation equipment

The source of gamma-irradiation used in this study was a 12,300 Curie Co^{60} Gamma Cell 220 designed by the Atomic Energy Canada Limited. The construction of the chamber of this radiator is shown in Fig. 1 (a) and (b). This equipment was convenient and suitable for irradiation in this research although the cobalt source had decreased in strength to approximately 4,745 Curie at the time of this investigation, hence, the lengthened irradiation time. This did not, however, affect the results as the growth of microorganisms was not involved.

2. Dose measurement

The Fricke-Miller ferrous sulphate dosimeter was used to determine the applied dose of irradiation. The application of this method is illustrated in Fig. 2 which shows the absorbances

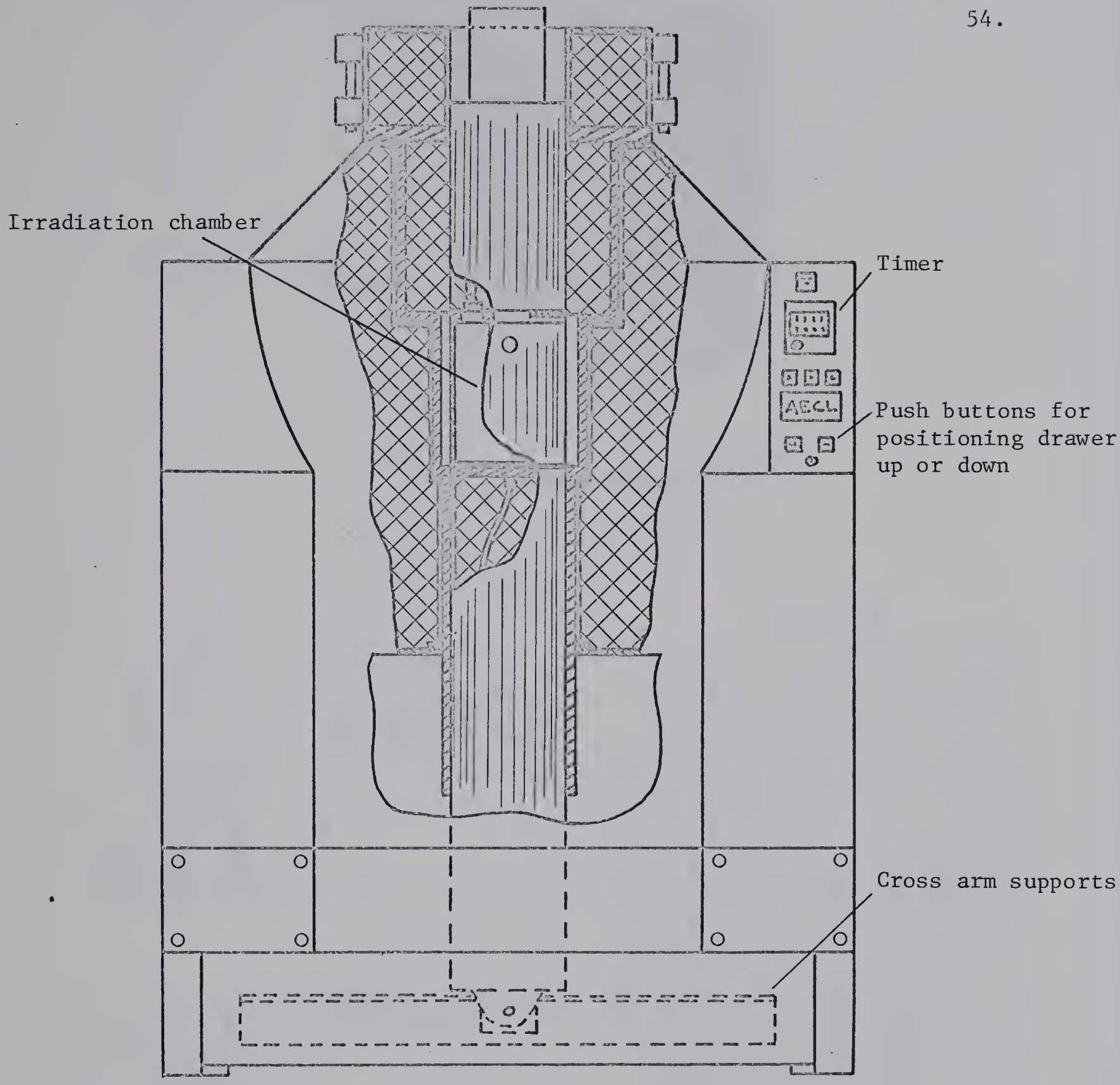
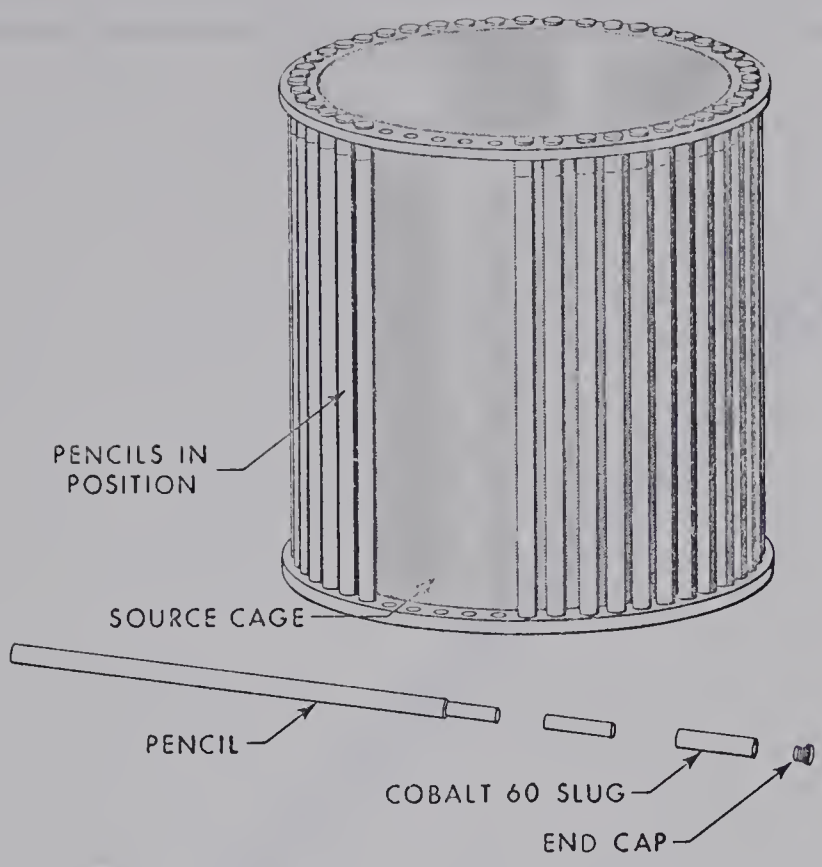
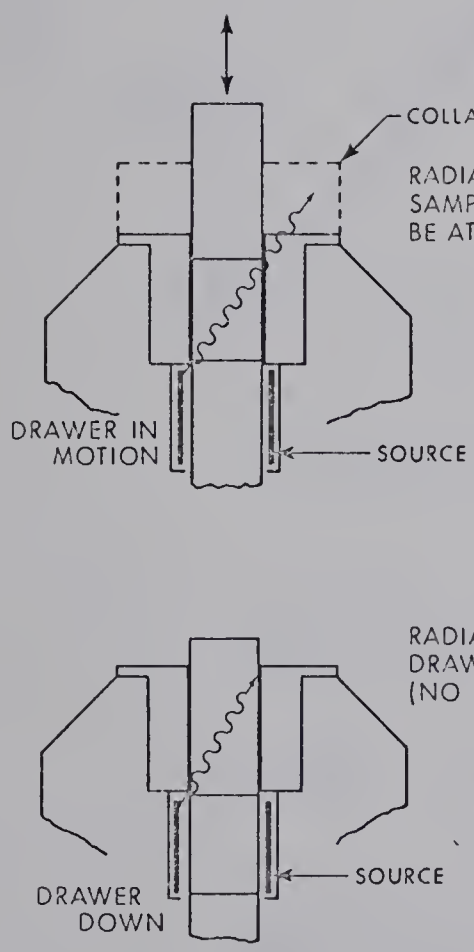


Fig. 1 (a) Diagram of Co^{60} Gamma-Cell 220 irradiation source used in this study





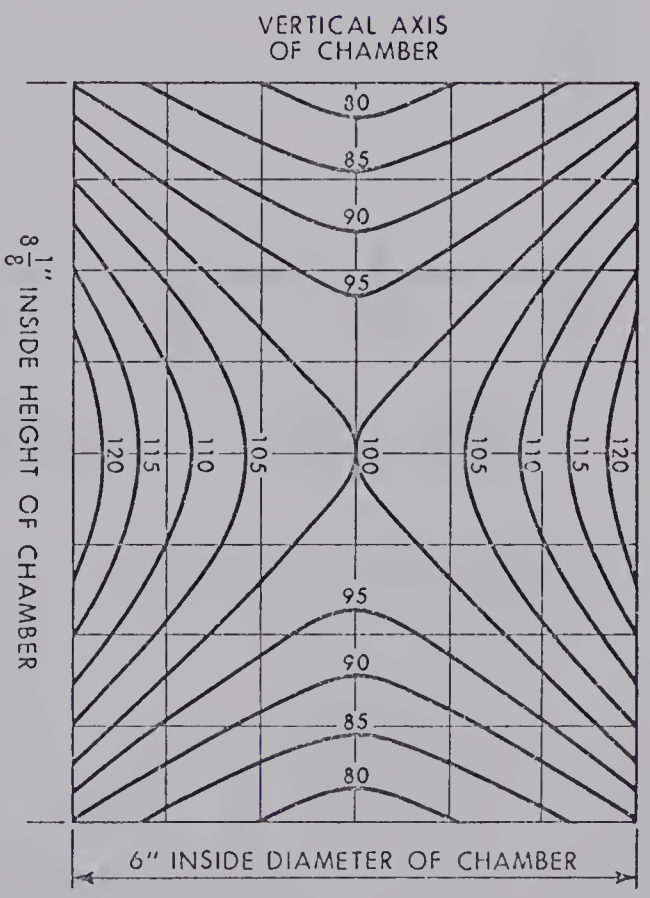


Fig. 1 (b) Diagram of Co^{60} irradiation source used in this study. The gamma-source cage and the isodose curves of the irradiation chamber

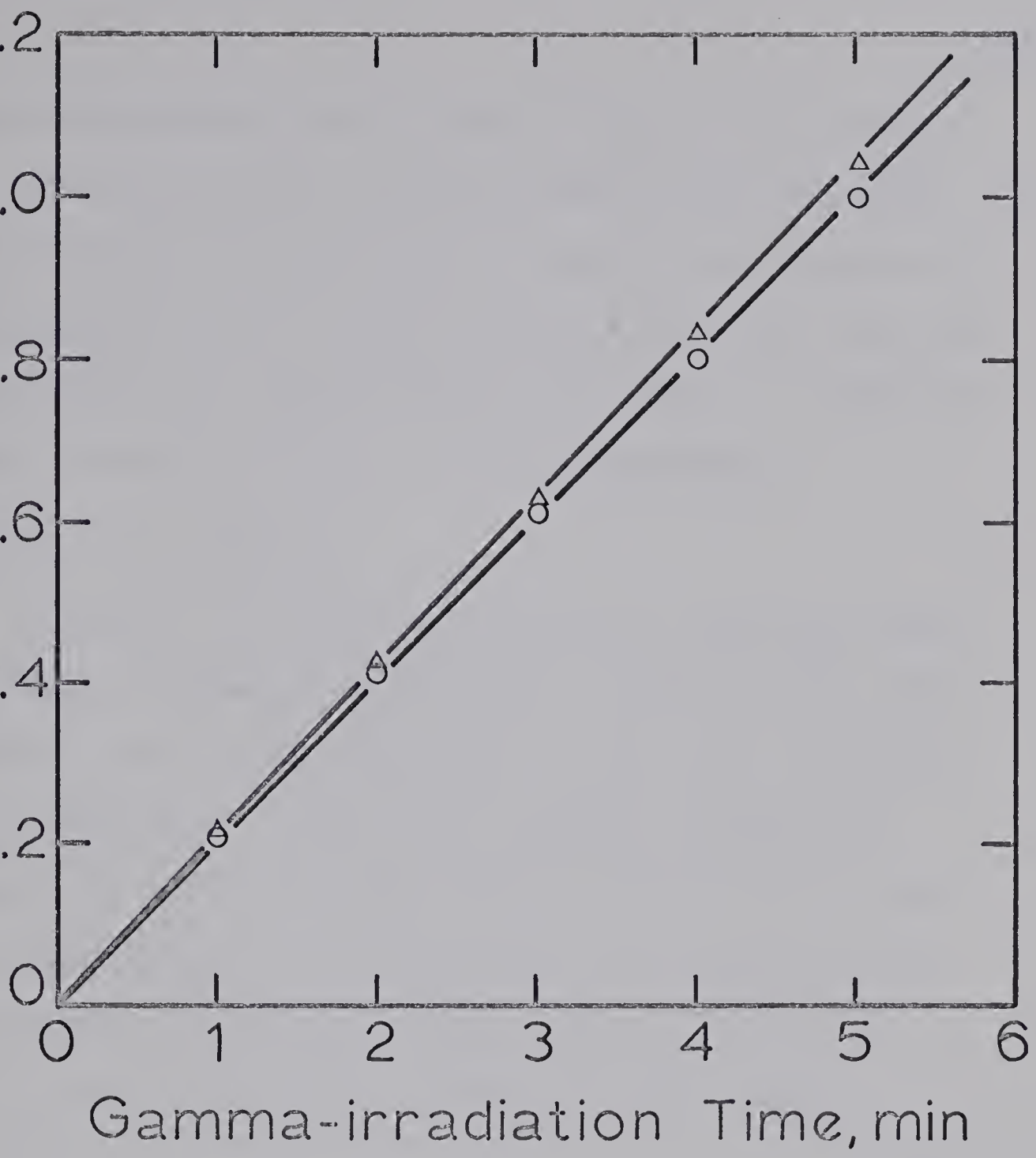


Fig. 2 Absorbance readings of dosimeter solution as a function of time of gamma irradiation

Legend: △——△ *Nov. 11 1969*

○——○ *March 20 1970*

Instrument used: Beckman Spectrophotometer Model DU (Cell thickness 1 Cm)

obtained on the standard ferrous sulphate solution by increasing exposure times on two dates during the conduct of this research. To obtain the actual dose rate the absorbances at 304 nm were used in the simplified equation: energy absorbed = $2.79 \times 10^4 \times A_{304}$ nm. The actual dose rates measured on November 11, 1969, and March 20, 1970, were 5.85 and 5.66 Krad per minute, respectively.

3. Procedure of irradiation

Commercial skimmilk powder without and laboratory dried skimmilk with the additives ascorbic acid, labelled ascorbic acid and ascorbyl palmitate were irradiated at least three repetitions at ambient temperature. Except where indicated otherwise the irradiation dose was in the range of 0.2 to 1.6 Mrad and the dose rate was approximately 0.34 Mrad per hour as the irradiation was carried out for a period over one year. After irradiation the samples in cans were stored below -20^{o} until used in further analysis.

C. Sulfhydryl Groups Determination

The total and free sulfhydryl groups of skimmilk powder were determined by the method described by Lyster (1964) for the determination of masked and free sulfhydryl groups in heated milk and milk powder. The procedure is based on the use of two -SH specific reagents (a) p-chloromercuribenzoate (PCMB) and (b) 5,5'-dithiobis-2-nitrobenzoate (DTNB). The latter is a redox indicator which produces a yellow color in the presence of -SH groups which is then measured spectrophotometrically at 412 nm.

1. Determination of total sulfhydryl groups

Skimmilk powder, 0.27 g was weighed and added in each of five test tubes (1.5 cm x 13.5 cm). Then to each tube were added increasing volumes of PCMB solution, the last tube used as blank containing an excess of this solution. Dilute urea citrate buffer was added to make the volume to 11 ml and was mixed thoroughly. Finally, 0.1 ml of DTNB solution was added and mixed again. The color developed in each tube was determined after 2 min. The readings obtained were plotted against the volume of PCMB solution as shown in Fig. 3. A straight line was drawn through the points and the intercept obtained on the abcissa determined the equivalent volume of PCMB which combined with the released sulfhydryl groups of the milk proteins.

2. Determination of free sulfhydryl groups

The free sulfhydryl groups in skimmilk powder consist of those initially present and those formed in the course of physical and chemical changes during processing and irradiation. The quantitative determination of the free sulfhydryl groups was the same as that of the total sulfhydryl groups except that urea citrate buffer was omitted since it was not required to effect unfolding of the protein structure. To make up the reaction volume to 11 ml, citrate buffer was used.

D. Determination of Ascorbic Acid and Degradation Products Induced by Irradiation

In the determination of ascorbic acid, a technique of

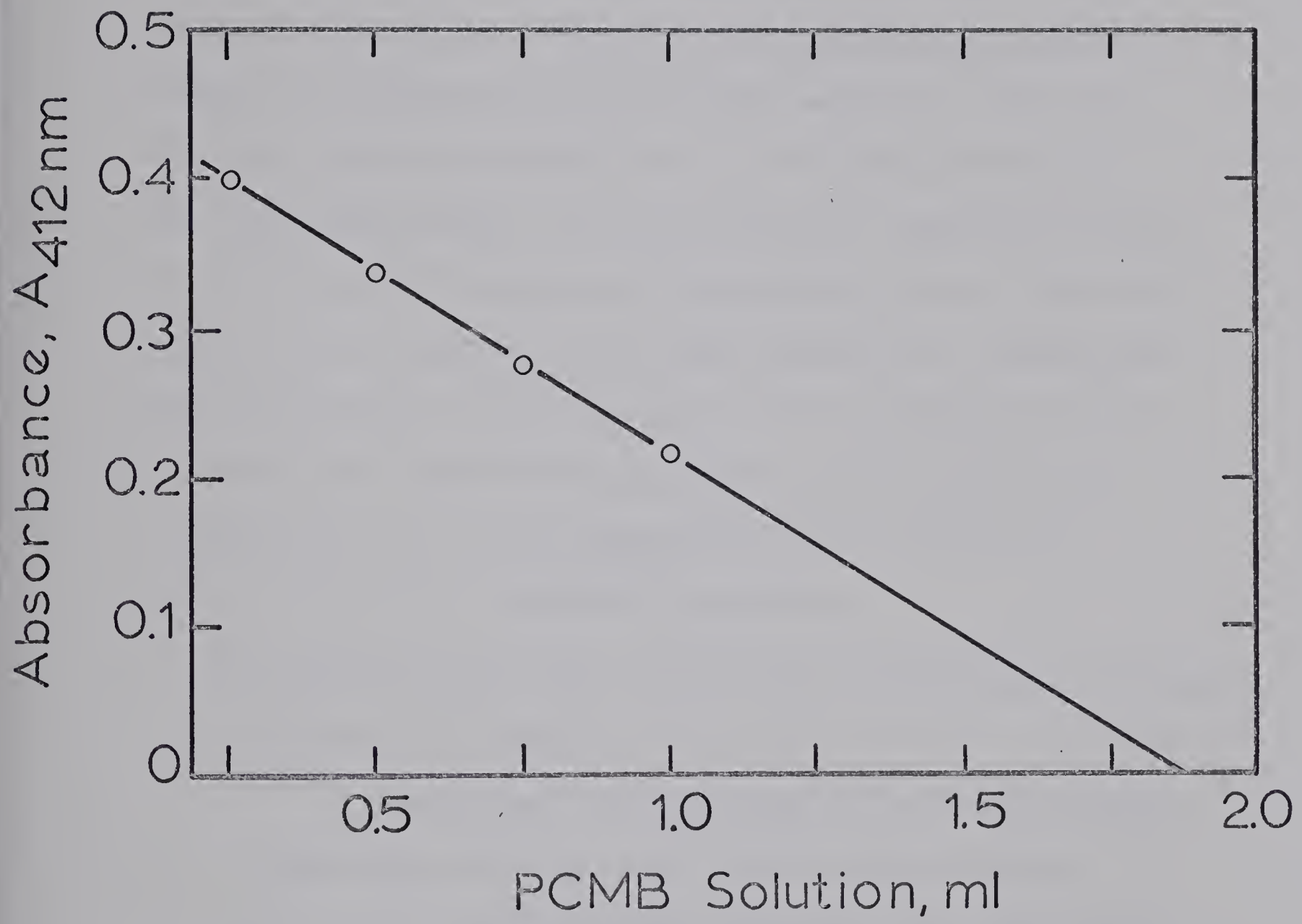


Fig. 3 The extrapolation graphical method used for determination of equivalent volume of PCMB

chromatographic separation was adopted from the "Methods of Vitamin Assay" by the Vitamin Chemists (1966). The technique has proven to be of value even in samples containing low concentrations of vitamin C. The vitamin was extracted by the usual way with the subsequent formation of red osazone. To remove the interfering degradation products of 2,4-dinitrophenylhydrazine the osazone was extracted with ethyl acetate and purified first by the column and then by thin layer chromatography. This method was also applied to recover the radioactive C^{14}- ascorbic acid from skimmilk powder. The radioactivity was then measured by thin layer radioactivity scanning and liquid scintillation counting using a standard liquifluor solution. In addition, gas liquid chromatography was applied in an attempt to characterize the volatile degradation products of vitamin C.

A brief outline of the procedure is as follows:

1. Determinations using column and thin layer chromatography techniques

   a. Extraction of vitamin C

      A slurry was made consisting of freeze-dried skimmilk powder containing 1 per cent ascorbic acid as carrier, 1.0×10^4 mC C_1^{14}- labelled ascorbic acid and 10 per cent metaphosphoric acid. The slurry was then diluted with 5 per cent phosphoric acid and mixed thoroughly. The suspended solids were removed by centrifugation and the supernatant obtained was collected. To this 2 to 3 drops of bromine were added and shaken gently until the solution was slightly yellow.

To remove the excess bromine, nitrogen was bubbled through the solution and finally 100 mg of thiourea was added per 20 ml of the solution.

For the formation of osazone, to a 10 ml aliquot of the above solution, 2 ml of 2 per cent 2,4 - dinitrophenylhydrazine reagent was added (Eastman Kodak). The reaction mixture was then kept for six hrs in a waterbath at 37°. To extract the osazones, 20 ml of ethyl acetate was used. The extract obtained was then evaporated under a stream of nitrogen to about 1 ml. To the residue, 10 ml of 3 per cent acetic acid in methylene chloride was added and the osazones were isolated by column or thin layer chromatography.

b. Separation of dinitrophenylhydrazone by column and thin layer chromatography

Chromatography column: The column was made of 40 g of silica gel of a particle size of 28 - 200 mesh (Fisher Scientific) which was suspended in 3 per cent acetic acid in methylene chloride. Chromatographic tube with dimensions of 2.2 cm ID x 37 cm length was used with glass wool plugged at the bottom. A uniform packing of the slurry was obtained by applying vacuum, leaving a small volume of the solvent to cover the column.

Thin layer plates: Silica gel G, 25 g, was mixed with 50 ml of water in a mortar and the slurry obtained was spread on plates (5 x 20 cm, 10 x 20 cm, and 20 x 20 cm) with a Shandon

type of spreader. The thickness of the layer was 250 μ. The plates were then dried at room temperature and activated in an oven at 110° for 2 hrs and stored until use in a desiccator.

Procedure: A solution of 3 per cent acetic acid in methylene chloride containing osazone solution (11 ml) was transferred to the column. Then the column was washed with 3 per cent acetic acid in methylene chloride until the mobile bands containing the excess phenylhydrazine and several other yellow derivatives were eluted. The ascorbic acid-phenylhydrazone remained on top of the column as an orange-red band. Then the column was washed with 2 per cent acetic acid in ethyl acetate and the fraction containing the orange-red band was collected. The effluent was then evaporated to dryness by using rotary flash evaporator. Then the residue was dissolved in 5 ml. ethyl acetate. The solution obtained was transferred into a 5 ml vial and then it was evaporated to exactly 2 ml under a stream of nitrogen. From this volume, 40 μl was applied to the thin layer plates. The plates were developed by a solvent system consisting of ethyl ether, ethyl acetate and glacial acetic acid (75:25:4 v/v). The separated osazone was identified by its Rf value.

c. Measurement of radioactivity

A scanning by applying a Geiger-Muller detector system and a scintillation counter was used.

Thin layer radioactivity scanning: Radioactivity of the C^{14}-ascorbic acid-hydrazone on thin plates was scanned by the Nuclear Chicago's thin layer conveyor system, Model 1006. The parameters of the measurements were as follows: high voltage - 1050 V, range 3 K/2, scan speed - 120 cm/hr and collimator speed - 3 mm.

Liquid scintillation counting: The silica gel containing the radioactive band was scraped into a scintillation vial and then was added 10 ml of a scintillation solution, made up of a solvent mixture of xylene 384 ml, dioxane 384 ml, absolute ethanol 231 ml containing 120 g napthalene, 4 g of 2,5-diphenyloxazole (PPO), and 0.5 g of p-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP) (Pilot Chemicals, Inc.). To stabilize the insoluble silica gel, 0.5 g of gelling agents, cab-o-sil (Packard Instrument Co. Inc., Downers Grove, Illinois) was incorporated into the scintillation solution.

2. Determinations using gas liquid chromatography technique

An aliquot of 28 g of 1.6 Mrad irradiated laboratory skimmilk powder containing 1 per cent of ascorbic acid was suspended in 80 ml of distilled water. So reconstituted skimmilk was then extracted 3 times with 250 ml portions of diethyl ether, the ether layer was decanted each time, the extracts combined and dried with anhydrous sodium sulfate. The extract was then evaporated to 2 ml by rotary flash evaporator at room temperature. The gas liquid

chromatographic analysis was carried out with this concentrated extract. Separations were achieved on a gas chromatography Bendix Model 2500 using U-shaped glass dual columns, having an OD of 0.89 cm and a length of 1.83 m. The inert solid support was Chromosorb P, acid washed, 80 - 100 mesh particle size, impregnated with 20 per cent of Carbowax 20 M (Mann Research Lab. Inc., N.Y.) as the liquid phase. The temperature programming applied was from 110 - 215^{o} at a rate of 1^{o} per min. The carrier gas was helium with a flow rate of 60 ml/min. Other parameters were input attenuation x 100, recorder attenuation x 1, suppress range x 100, detector used, hydrogen flame ionization.

E. Gas Liquid Chromatography of Irradiated Skimmilk Powder Volatiles

1. Isolation of volatiles

The procedure for recovery of volatiles from both irradiated and control commercial high heat skimmilk powder samples was as follows: 600 g of skimmilk powder in a 5 liter round bottom flask was suspended with 1800 ml of distilled water plus 30 ppm of Dow - Corning Antifoam AF. The flask was immersed in cold water and then attached to the trapping system (see Fig. 4). The air pressure in the flask was lowered to 6 mm Hg with an oil vacuum pump and nitrogen gas was allowed to bubble through the sample, thus providing agitation of the suspension and inert atmosphere in the apparatus. Then the content was heated to 40^{o}. Approximately 100 ml of total

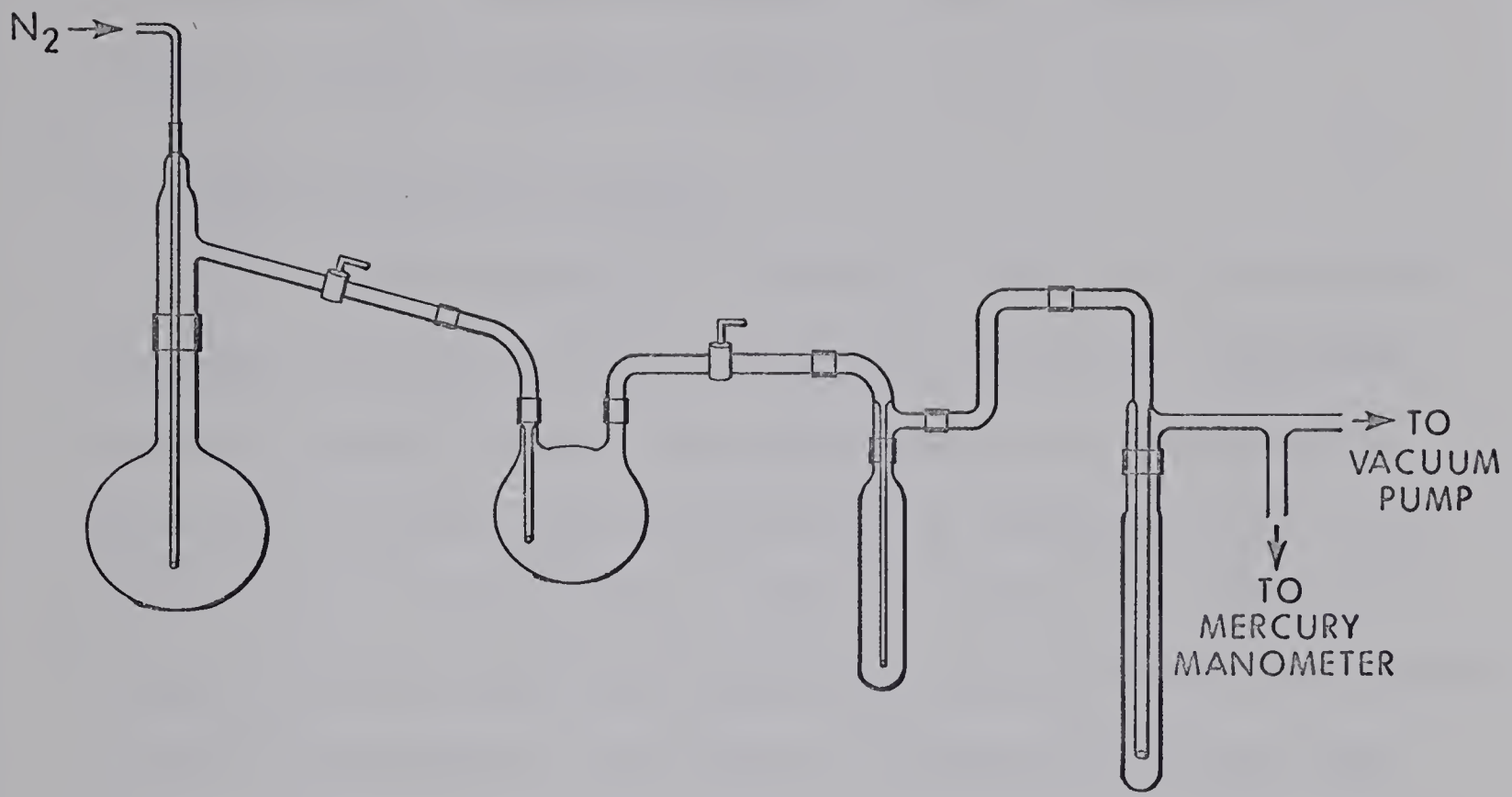


Fig. 4 Low-temperature-reduced-pressure distillation apparatus

1. *Distillation flask (5-liters)*
2. *Ice-water trap*
3. 4. *Liquid nitrogen traps*

distillate was collected during 4 hrs of distillation, the most of it condensing in the ice-water trap. For the non-irradiated samples, 4 aliquots each of 600 g was distilled in order to obtain sufficient amounts of sulfur-containing volatiles.

2. High boiling point volatiles

Aqueous solution of the volatiles collected in an ice-water trap were extracted at 4^{o} with 100 ml of diethyl ether. The extraction was repeated 4 times. The ether extracts were combined and dried with anhydrous sodium sulfate and then evaporated at 0^{o} to 2 ml with a rotary flash evaporator. Then 2 µl of the ether extract was injected into the Bendix Gas Chromatograph using as previously U-shaped columns of Chromosorb P, acid washed, impregnated with 20 per cent Carbowax 20 M as a stationary phase. Temperature programming was applied from 100 to 200 at a rate of 8^{o} per min. The other parameters applied were temperature of detector thermostat, 230^{o}, and the injector space temperature, 210^{o}.

3. Low boiling point sulfur-containing volatiles

These volatiles were collected in a liquid nitrogen trap. After the end of distillation 3 per cent of aqueous solution of mercuric chloride was added to precipitate the sulfur-containing compounds. The precipitate formed was separated from the supernatant by centrifugation. A portion of the precipitate was transferred into a 25 ml screw capped scintillation vial fitted internally with

a septum and attached to the entrainment assembly (see Fig. 5 (a)). This assembly consisted of two syringe needles inserted into the vial, the first, inlet needle and the second, outlet needle, were held rigidly parallel by the Leuer-Lock fittings soldered to a stainless steel plate. Through this inlet and outlet the entraining gas was conducted into and from the sample vial to the gas sampling valve.

To generate the sulfur-containing volatiles from the precipitate 0.5 ml of 6 N hydrochloric acid was injected into the vial which was heated to 135^{o}. Then, helium was used to flush the vial at a rate of 30 ml/min and the volatiles released were carried into the helical trap immersed in liquid nitrogen through the gas sampling valve. This valve controlled the flow of volatile and carrier gas. By pushing in the valve the carrier gas and volatiles were carried into the helical trap and when the valve was pulled out, the flow of the gas from the vial was blocked while the volatiles trapped in the helical trap were carried through the heating block into the gas chromatography column.

Attached to the heating block and connected to the gas chromatograph was a pair of toggle valves. One toggle valve, valve No. 1 (see Fig. 5 (b)) controlled the inlet of the internal carrier gas (the carrier gas flow of the gas chromatograph) and the second, valve No. 2, controlled the inlet of the volatiles into the gas chromatograph from the attached helical trap.

After all the volatiles have been trapped into the helix

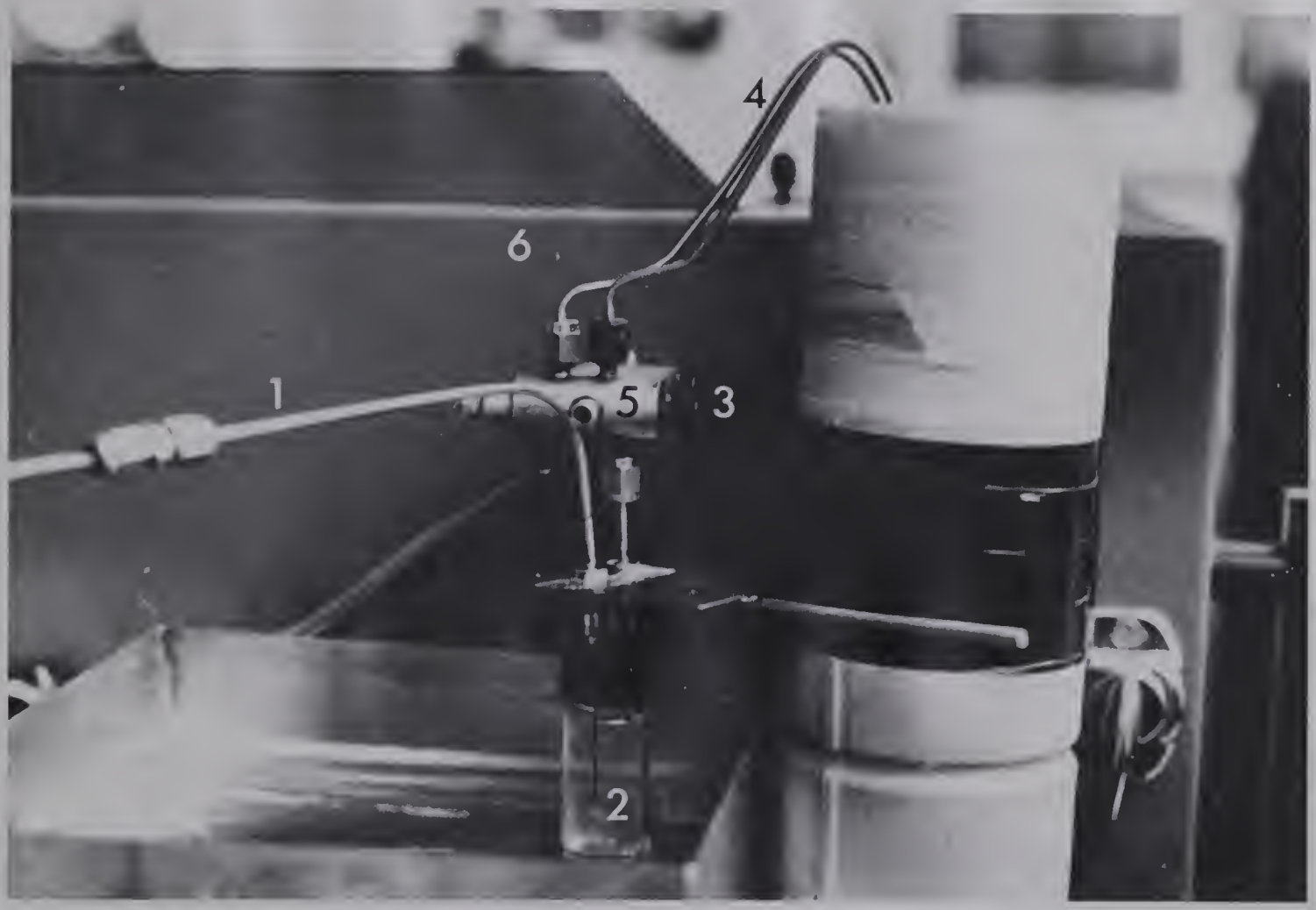


Fig. 5(a) On column flush chromatography attachment used for sulfur containing volatiles. The assembled entrainment system.

1. *The external carrier gas*
2. *The screw-capped scintillation vial*
3. *The gas sampling valve*
4. *The helical trap*
5. *The inlet of gas sampling valve*
6. *The gas chromatograph*

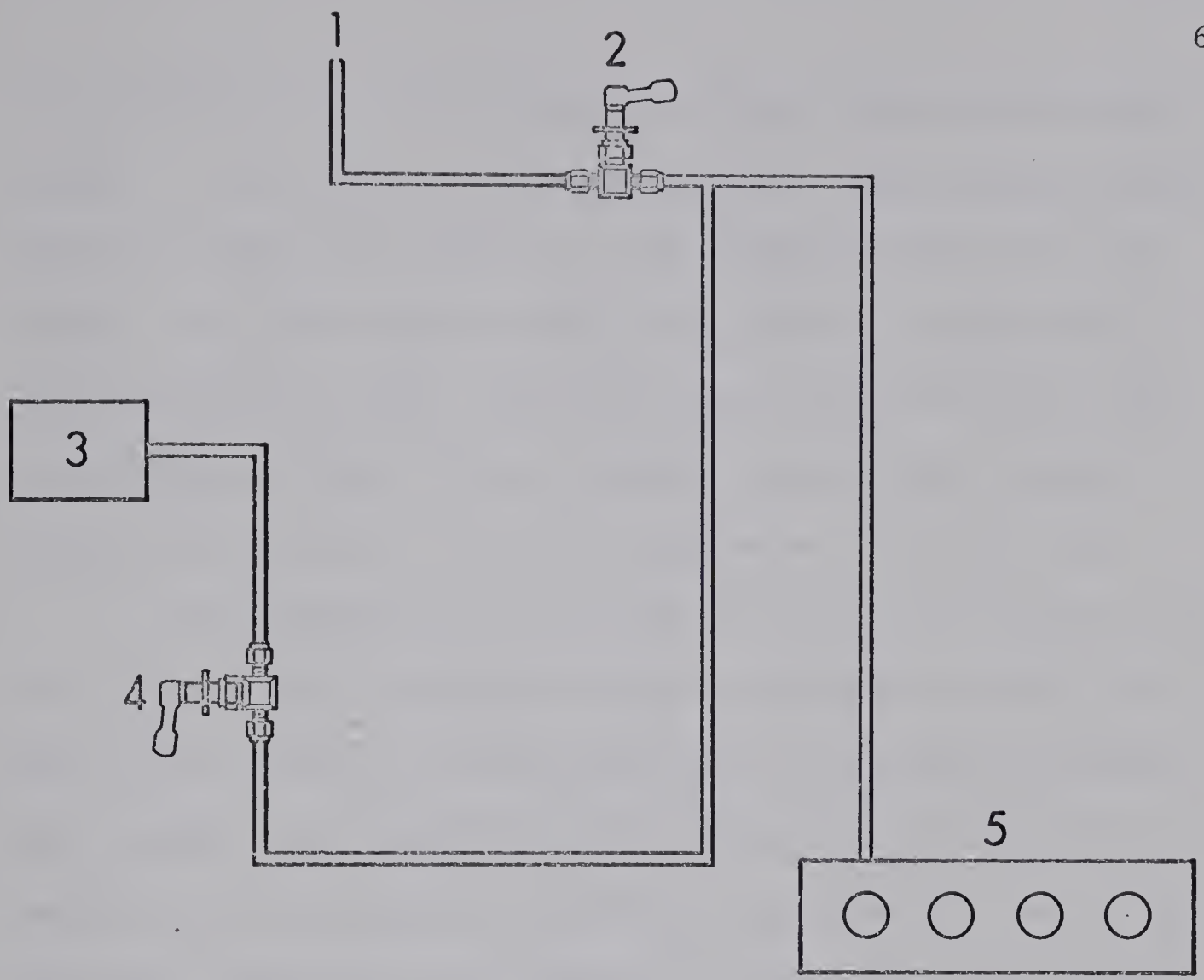


Fig. 5 (b) *On column flush chromatography attachment used for sulfur-containing volatiles. The toggle valves of the helium line*

1. *The internal carrier gas (the carrier gas flow of the GLC)*
2. *Toggle valve No. 1.*
3. *Heating block with the assembled entrainment system*
4. *Toggle valve No. 2*
5. *Injection port*

the external helium flow line was transferred from the entrainment assembly to the inlet of gas sampling valve. When the gas sampling valve was pulled out, valve No. 1 was closed and valve No. 2 was opened. Then liquid nitrogen gas was removed and replaced with an oil bath heated at 135°. After one minute toggle valve No. 2 was simultaneously closed and the volatiles transferred to the gas liquid chromatograph and further separated as is usually done.

The separation was achieved by heating the column at 50° for 4 min and then the temperature was programmed from 50 to 145° at a rate of 4°/min. As indicated earlier, U-shaped dual columns were applied using Firebrick as inert solid support 60 to 80 mesh particle size, impregnated with 30 per cent Apiezon M (Associated Electrical Industries Ltd., England) as stationary liquid phase. For identification of individual compounds co-chromatography technique was applied using the following chemically pure mercaptans and sulfides: ethyl - , iso-propyl - , t-butyl - , allyl - , n-propyl - , sec-butyl - , iso-butyl - , n-butyl - , t-amyl - , and isoamyl mercaptans: dimethyl - , ethylmethyl - , and diallyl sulfides (PolyScience Co., Evanston, Illinois).

F. Amino Acid Analysis of Casein Isolated from Irradiated Skimmilk Powder

1. Isolation of casein

Non-irradiated and irradiated skimmilk powder were reconstituted with distilled water (97 g/291 ml water). The reconstituted skimmilk was adjusted to pH 4.5 by titration with 1 N hydrochloric acid at room temperature. The fluffy precipitate of the

casein was isolated by centrifugation and the precipitate was washed 3 times with distilled water using centrifugation each time to recover the precipitate. The washed casein was then adjusted to pH 8.0 by addition of 1 N sodium hydroxide and reprecipitated by addition of 1 N hydrochloric acid to pH 4.6. After repeated washing with distilled water, the casein was freeze dried and stored at 4°.

2. Analysis of amino acids

Duplicate samples of casein, 0.8 g, were hydrolyzed with a 1:1 v/v mixture of 98 per cent formic acid and concentrated hydrochloric acid, approximately 6 N, for 20 hrs by gentle reflux at 120°. Hydrolysis was carried out under an atmosphere of nitrogen by bubbling the gas through the solution. Then the sample was made up to 100 ml with deionized water, shaken well and filtered. The clear solution was evaporated to dryness with a rotary flash evaporator and after adding 100 ml of deionized water the evaporation was repeated. The residue was then made up again to 100 ml with water and the solution so obtained was adjusted to pH 2. Before amino acid analysis, 5 ml of this hydrolyzate was diluted to 10 ml with "Michalis" buffer, pH 2.2 and a volume of 0.1 ml was applied to the Spinco Amino Acid Analyzer.

The cystine and cysteine were analyzed separately from samples hydrolyzed for 7 instead of 20 hrs, by the method of Wallace and Aiyar (1968). The pink color developed by ninhydrin reagent was read at 575 nm using a Beckman spectrophotometer Model DBG.

IV. RESULTS

A. The Effect of Irradiation on the Sulfhydryl Groups

1. Amino acid composition and sulfhydryl group content in casein

The amino acid composition of the isolated and hydrolyzed casein from 25, 50 and 100 Krad irradiated and non-irradiated commercial skimmilk powder is presented in Table 1. The results obtained are an average of at least two determinations. The total amino acid content decreased with increasing doses of irradiation in amounts of 3.6, 0.3 and 9.0 per cent, respectively, as compared to control. The amino acids that were affected by irradiation at the highest dose applied were half cystine, isoleucine, arginine, serine and threonine, with reductions of 22, 17, 14, 14 and 11 per cent, respectively. At the lowest dose applied, i.e. 25 Krad, half cystine, arginine and histidine decreased by 22, 18 and 13 per cent, while at 50 Krad only half cystine has shown a decrease as compared with the amount found in non-irradiated samples serving as a control. Contrary to expectation methionine and some other irradiation sensitive amino acids were not changed after irradiation at the three dose levels applied.

As seen in Table 2, the cystine content of skimmilk powder decreased after irradiation at 25 Krad. At increasing irradiation doses the cysteine content increased and at 100 Krad it was at the highest value of 201 mg/100 g of dry casein. Contrary to this finding the content of cystine decreased at all doses. Again

Table 1 - Amino acid composition of casein isolated from non-irradiated and irradiated commercial skimmilk powders exposed to doses of 25, 50 and 100 Krad

	Non-irradiated sample (control)	Irradiation dose in Krad		
		25	50	100
	Amino acid content as per cent of total amino acids *			
Lysine	7.82	7.19	7.50	7.07
Histidine	2.63	2.28	2.48	2.51
Arginine	3.53	2.91	3.50	1.57
Aspartic acid	6.98	6.76	7.45	6.59
Threonine	3.90	4.32	4.02	3.46
Serine	4.45	4.58	4.69	3.84
Glutamic acid	21.04	23.57	20.93	19.61
Proline	9.85	8.87	9.61	9.55
Glycine	1.77	1.63	1.76	1.72
Alanine	3.25	2.96	3.16	3.16
Half cystine	1.23	0.95	0.99	0.96
Valine	5.99	5.38	6.05	5.44
Methionine	2.63	2.54	2.60	2.45
Isoleucine	4.70	4.32	4.58	3.89
Leucine	9.46	8.75	9.36	8.67
Tyrosine	4.81	5.00	5.03	4.67
Phenylalanine	4.52	3.10	4.61	4.35
Ammonia	1.40	1.27	1.34	1.48
Total	99.96	96.38	99.66	90.97

** The results are an average of at least two determinations*

Table 2 - The content of cysteine and cystine in casein of irradiated skimmilk powder

Irradiation Dose Krad	Contents of cysteine and cystine mg / 100 g dry casein*		
	Cysteine	Cysteine after reduction	Cystine by difference
Control	186	363	177
25	177	314	136
50	184	336	153
100	201	307	106

* *The results are an average of at least two determinations*

contrary to expectation the dose of 25 Krad had a more pronounced effect than the next dose of 50 Krad. Theoretically the content of cystine recovered after irradiation should be closely related to the content of cystine present in the milk sample before irradiation. As seen in Table 2, such relationship is not revealed in the casein isolated from irradiated skimmilk powder at 50 Krad, which result could indicate that apart from the simple homolytic cleavage of cystine into cysteine at higher irradiation doses applied, other processes might occur which are not simply reflected by the cysteine and cystine contents of casein.

2. The change of sulfhydryl groups in skimmilk powder

The freeze-dried laboratory skimmilk powder obtained in our laboratory consisted of large, flat, fluffy particles compared with the commercial skimmilk powder which had fine, dense, creamy colored particles. The former powder turned light brown after irradiation although the color produced was not as intense as that of the commercial sample. The color change was most pronounced when both skimmilk powders were irradiated at the highest dose applied, e.g. 1.6 Mrad.

The changes of -SH groups in commercial and laboratory prepared skimmilk powders, as related to the irradiation dose applied, are presented in Fig. 6 and Tables 3 and 4. As presented in Table 3, gradual increases in irradiation dose level from 0.2 to 1.6 Mrad caused changes in free, masked and total -SH groups. There was a trend for the free or unmasked -SH content

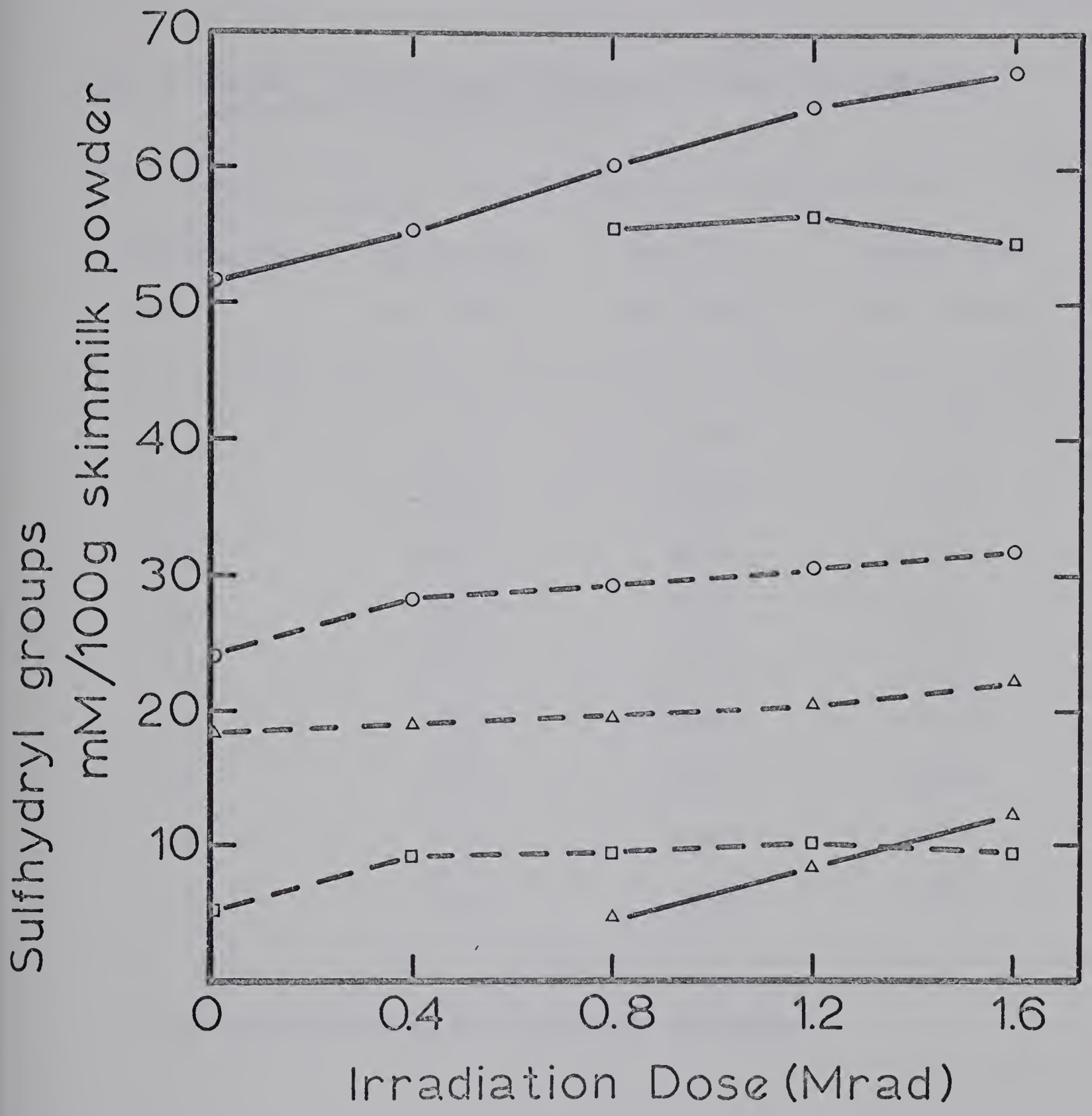


Fig. 6. Total, free and masked sulfhydryl groups in commercial and laboratory skimmilk powders

Legend: ○ *Total -SH* □ *Masked -SH*

△ *Free -SH*

Broken line - Commercial skinmilk powder
Solid line - Laboratory skimmilk powder

Table 3 - Total, free and masked sulfhydryl groups in irradiated commercial skimmilk powders

Irradiation dose Mrad	Total -SH mM / 100 g	Free -SH mM / 100 g	Masked -SH* mM / 100 g
Control	24.07	18.52	5.55
0.2	24.44	19.63	4.81
0.4	28.14	18.88	9.26
0.6	30.36	19.53	10.83
0.8	29.25	19.63	9.62
1.0	30.36	19.26	11.10
1.2	30.73	20.37	10.36
1.4	31.18	21.33	9.85
1.6	31.85	22.20	9.65

* *From difference of total and free -SH groups*

Table 4 - Total, free and masked sulfhydryl groups in laboratory skimmilk powder

Irradiation dose Mrad	Total - SH mM / 100 g	Free - SH mM / 100 g	Masked - SH* mM / 100 g
Control	51.85	-	51.85
0.4	55.18	-	55.18
0.8	60.37	4.81	55.56
1.2	64.81	8.14	56.67
1.6	67.03	12.22	54.81

* *From difference of total and free - SH groups*

to increase with increasing irradiation dose. Up to 1 Mrad, this value was an average of 19.5 mM/100 g of skimmilk powder, while at about 1 Mrad and up to 1.6 Mrad the free sulfhydryl groups increased stepwise by 1 per cent for each increase of 0.2 Mrad.

When the estimation of sulfhydryl groups was made in the presence of urea, a very effective protein unfolding agent, the sulfhydryl content was found to be higher by an average of 10 mM/100 g of skimmilk powder. It should be emphasized that an irradiation dose increase induced also a slight rise in content of total sulfhydryl groups, which should indicate a disulfide bond rupture within the protein molecules. As revealed by the content of the masked sulfhydryl groups, at initial irradiation doses the disulfide rupture increased the masked portion of the total content of sulfhydryl groups while at the higher doses applied the free sulfhydryl group portion was enriched. The difference between the total and free sulfhydryl groups was calculated and presented as the content of masked sulfhydryl groups. The rationale for this was that only the freely exposed sulfhydryl groups were available for reaction with analytical reagents used, while those within the protein globules were sterically hindered or covered and did not react. By adding urea, a total unfolding of protein aggregates was induced and additional sufhydryl groups became readily accessible to the analytical reagents used.

From Table 4, it can be seen that the content of total, free and masked sulfhydryl groups of the laboratory prepared

skimmilk powder differed markedly from that of the commercially prepared powders. The amount of the readily accessible sulfhydryl groups, i.e. free -SH groups was zero in the control sample and that in the irradiated samples it was much lower than that found in the skimmilk powders prepared commercially. On the other hand, there was a higher content of total -SH groups, both in control and irradiated samples in laboratory prepared skimmilk powder. Consequently, the content of masked sulfhydryl groups was also higher. These differences in the sulfhydryl groups in laboratory and commercially produced skimmilk powders amounting to as much as 100 per cent appear to be caused by the differences in the processing history of the two powders. High heat treatments of the milk exposed to drying processes could lower the milk total sulfhydryl groups and consequently both free and masked sulfhydryl groups would also be affected. The laboratory skimmilk powder was pasteurized and dried on the same day, and high temperature treatment was avoided through the use of freeze-drying technique, i.e. vacuum of 10 μ and a temperature at the beginning of drying of -40° that only rose to 50° at the end of freeze-drying operations. In addition to a marked difference observed in the content of the total -SH groups in control samples of commercially and laboratory prepared skimmilk powders there was a marked difference in the increase of total -SH groups induced by irradiation. While the commercial samples exposed to irradiation doses about 0.8 Mrad were characterized by a slight increase of total -SH groups, less

than 1 mM per 0.2 Mrad, in laboratory skimmilk powder the increase was twice as high.

B. The Effect of Irradiation on the Volatiles of Skimmilk Powder

1. High boiling point volatiles

From the low-temperature and reduced-pressure distillation of the control, non-irradiated skimmilk powder and skimmilk powder irradiated at the highest dose, i.e. 1.6 Mrad, two fractions of volatiles were collected: (a) a high boiling point distillate collected in an ice-water trap and (b) low boiling point compounds which were collected in the liquid nitrogen trap. Qualitative tests were conducted on the volatiles collected in both condensates using a 3 per cent mercuric chloride solution. No sulfur-containing compound was found in the condensate collected in the ice-water trap, but a fluffy, white precipitate formed in the condensate of the liquid nitrogen trap due to the presence of sulfur-containing compounds.

The ice-water condensate extracted with diethyl ether was further separated by gas liquid chromatography. The chromatograms obtained after injection of approximately 4 µl of diethyl ether extracts are presented in Fig. 7. As seen from the chromatograms of irradiated samples, 30 compounds could be separated while the non-irradiated samples contained 12 fewer components, i.e. 18 compounds. The volatile compounds from the irradiated skimmilk powder designated with peak numbers 2, 3, 4, 5, 6, 7, 9, 17, 22,

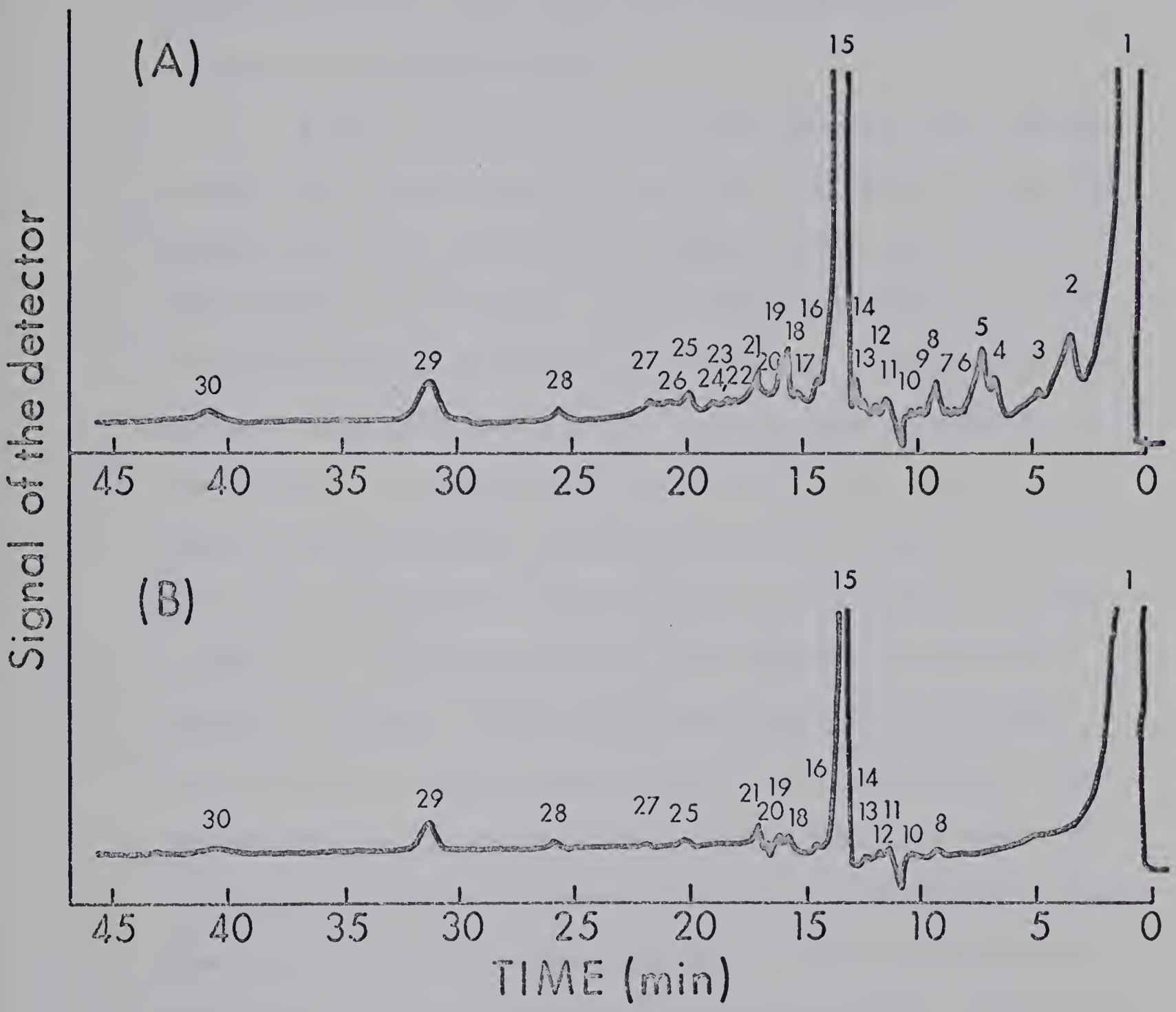


Fig. 7 *The chromatogram of volatiles from ice-water condensate of irradiated (1.6 Mrad) (A) and non-irradiated (B) commercial skimmilk powder.*

Gas chromatograph: *Bendix Model 2500*

Liquid phase: *20 per cent of Carbowax 20 M on 80 - 100 mesh acid-washed Chromosorb P*

Carrier gas flow rate: *30 ml/min of helium*

Sensitivity: *Input attenuation,* x 100; *Recorder attenuation,* x 1; *Suppression range,* x 100

Temperature programming: *At 100° for 3 min and then programmed to 200° C at a rate of 8°/min*

23, 24 and 26 did not appear in the non-irradiated samples. These components were tentatively assumed to be volatiles of carbonyl compounds, alcohols, lower fatty acids and hydrocarbons.

2. Sulfur-containing volatiles

A total of 20 sulfur-containing compounds were separated by gas liquid chromatography in the 1.6 Mrad irradiated commercial skimmilk powder as compared to 12 compounds separated from the non-irradiated samples (Fig. 8). The retention times of all the compounds separated in both the irradiated and non-irradiated samples are listed in Tables 5 and 6. In an attempt to identify the nine additional compounds found in the irradiated samples a gas liquid co-chromatography technique was applied using analytical grade sulfur compounds of low and medium boiling points. In Fig. 9 under (A), a chromatogram of the pure reference sulfur compounds is presented. Some of the peaks separated corresponded by the retention times to those found in the chromatogram of the volatiles of the irradiated skimmilk (see chromatogram under B). The tentative identity of these volatiles was confirmed by chromatograms which presented a co-chromatogram of volatiles from sample with added pure sulfur compounds (see chromatogram C). From these comparisons the additional eight sulfur compounds formed by irradiation, were ascribed to ethyl mercaptan, allyl mercaptan, n-propyl mercaptan, sec-butyl mercaptan, n-butyl mercaptan and diallyl sulfide. The compounds which were not found in the non-irra-

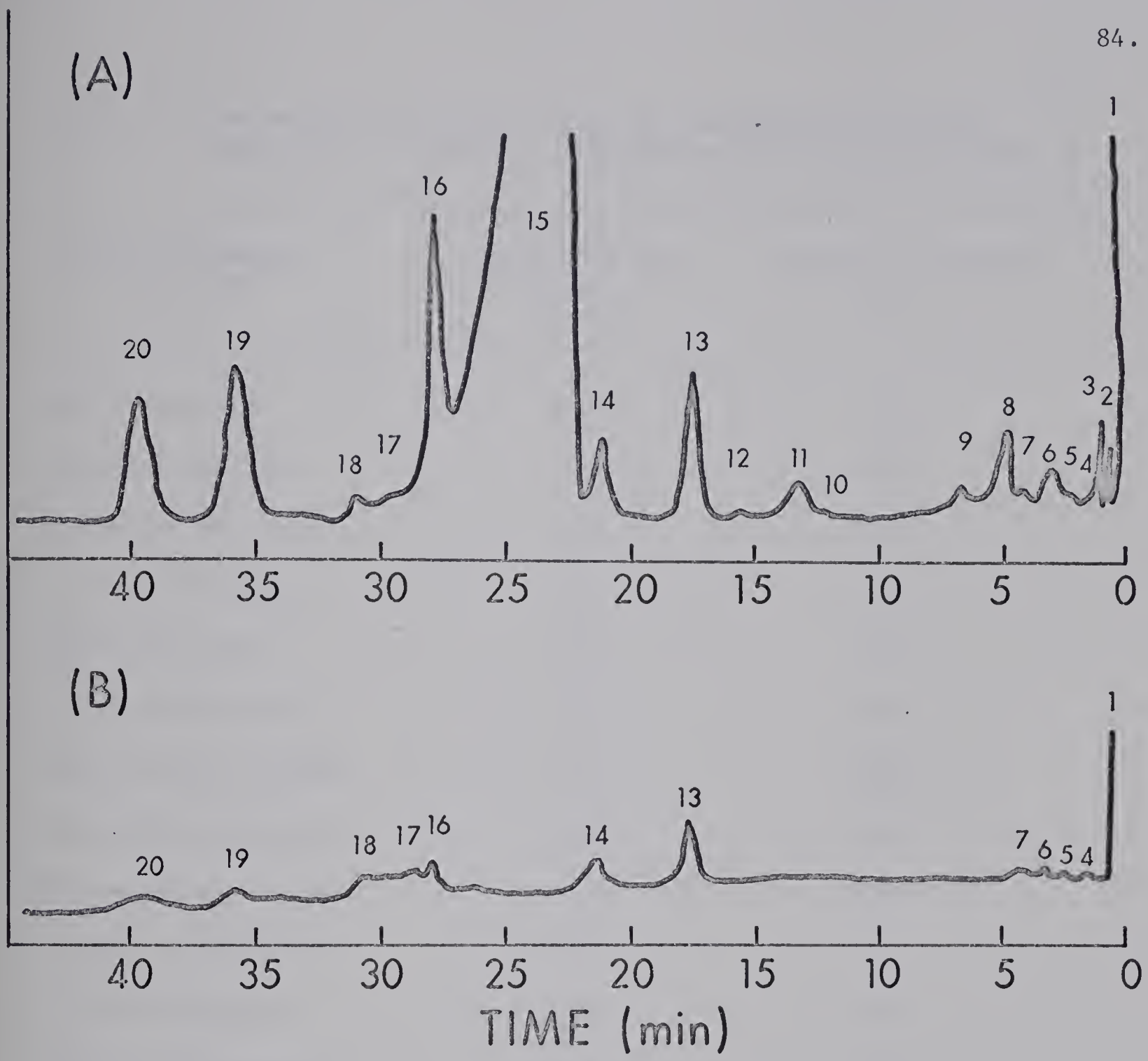


Fig. 8 The chromatogram of sulfur-containing volatiles recovered in liquid nitrogen trap from irradiated (1.6 Mrad) (A) and non-irradiated (B) commercial skimmilk powder

Gas chromatograph: Bendix Model 2500

Liquid phase: 30 per cent of Apiezon M on 60 - 80 mesh Firebrick
Carrier gas flow rate: 30 ml/min of helium
Sensitivity Input attentuation, x 100; *Recorder attenuation,* x 1, *Suppression range,* x 100
Temperature programming: At 50° C for 4 min and then programmed to 145°C at a rate of 4°/min

Table 5 - The relative retention times of some analytical grade mercaptans and sulfides (PolyScience Co., Evanston, Ill.)

Sulfur-Containing Compounds	Retention Time (min)	Relative Retention Time
Ethyl Mercaptan	6.4	0.32
Dimethyl Sulfide	7.2	0.34
Isopropyl Mercaptan	9.6	0.48
t-Butyl Mercaptan	11.5	0.58
Allyl Mercaptan	11.6	0.58
n-Propyl Mercaptan	13.0	0.65
Ethyl Methyl Sulfide	13.3	0.66
Sec-Butyl Mercaptan	16.8	0.84
Iso-Butyl Mercaptan	17.7	0.88
n-Butyl Mercaptan*	20.0	1.0
t-Amyl Mercaptan	20.1	1.0
Iso-Amyl Mercaptan	22.9	1.14
Diallyl Sulfide	28.2	1.41

* *n-Butyl Mercaptan chosen as reference (=1)*

Gas Chromatograph Bendix Model 2500

Liquid phase: 30 per cent Apiezon M on 60 - 80 Mesh Firebrick
Carrier gas flow rate: 30 ml/min of helium
Sensitivity: Input attenuation, x 100; Recorder attenuation, x 50
Suppression range, x 10K
Temperature programming: At $50^{o}C$ for 4 min and then programmed to $145^{o}C$ at a rate of 4^{o}/min

Table 6 - Retention times in mins of the volatile sulfur compounds formed in commercial skimmilk powder irradiated at 1.6 Mrad

Peak No.	Non-irradiated sample (control)	Irradiated
1	0.7	0.7
2		0.8
3		1.1
4	1.6	1.6
5	2.3	2.4
6	3.1	3.1
7	4.2	4.3
8		4.9
9		6.7
10		12.4
11		13.3
12		15.6
13	17.5	17.4
14	21.2	21.2
15		23.4
16	27.8	27.8
17	29.4	29.8
18	30.8	30.8
19	35.6	35.6
20	39.0	39.5

GLC separation data as in Figure 8

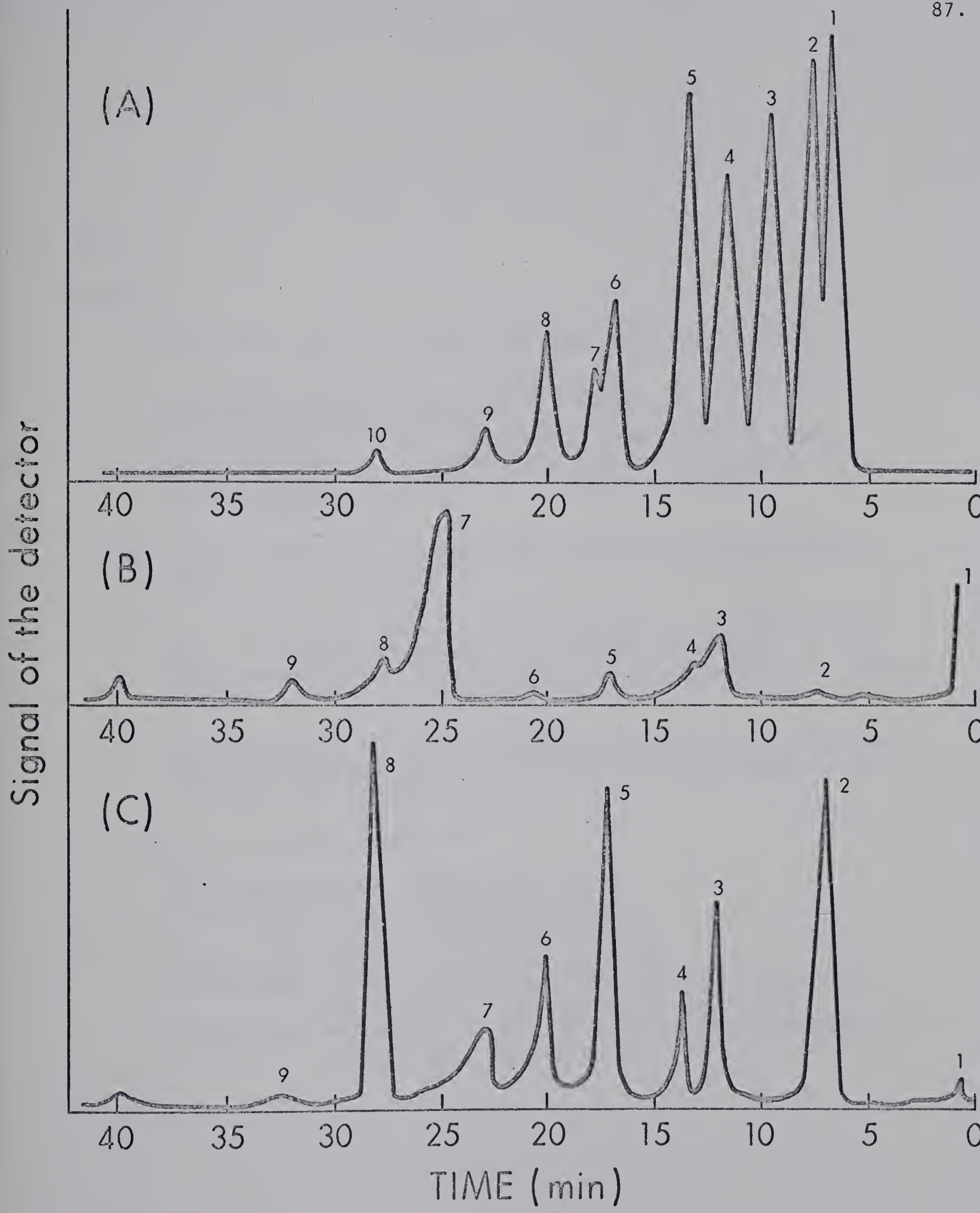


Fig. 9 *GLC of analytical grade mercaptans and sulfides and their co-chromatograms with sulfur-containing volatiles of irradiated skimmilk powder*

Fig. 9

Legend:

A. Analytical grade mercaptans and sulfides (Evanston Ill)

1. *Ethyl mercaptan* 2. *Dimethyl sulfide* 3. *Iso-propyl mercaptan (t-Butyl mercaptan)* 4. *Allyl mercaptan* 5. *n-Propyl mercaptan (Ethyl Methyl disulfide)* 6. *Sec-Butyl mercaptan* 7. *Iso-Butyl mercaptan* 8. *t-Amyl mercaptan (n-Butyl mercaptan)* 9. *Iso-Amyl mercaptan* 10. *Diallyl sulfide*

B. Sulfur-containing volatiles from commercial skimmilk powder irradiated at 1.6 *Mrad.*

1. *Hydrochloric acid* 2. *Ethyl mercaptan* 3. *Allyl mercaptan* 4. *n-Propyl mercaptan* 5. *Sec-Butyl mercaptan* 6. *n-Butyl mercaptan* 7. *Dimer of mercaptans or sulfides* 8. *Diallyl sulfide*

C. Co-chromatogram of sulfur-containing compounds from irradiated commercial skimmilk powder and analytical grade compounds of mercaptans and sulfides

Identity of peaks as under B

Gas chromatograph: *Bendix Model 2500*
Liquid phase: *30 per cent of Apiezon M on 60 - 60 mesh Firebrick*
Carrier gas flow rate: *30 ml/min of helium*
Sensitivity: *Input attenuation,* x 100; *Recorder attenuation, A,* x 10 *B,* x 2; *Suppress range,* x 10K
Temperature programming: *At* 50^{o} *for 4 min and then programmed to* 145^{o} *at a rate of* 4^{o}*/min*

diated sample were ethyl mercaptan, allyl mercaptan and n-propyl mercaptan. It should be emphasized that the use of 30 per cent Apiezon M as liquid phase on 60 - 80 mesh firebrick as inert solid support, t-butyl mercaptan and allyl mercaptan had the same retention times. This applies similarly to n-butyl and t-amyl mercaptans.

Dimethyl sulfide, iso-propyl mercaptan, t-butyl mercaptan, ethyl methyl sulfide, iso-butyl and iso-amyl mercaptans all analytical grades were also used in the co-chromatography study and as revealed from corresponding chromatograms these compounds were not present in either the irradiated or non-irradiated powders.

C. The Effect of Additives on the Irradiation of Skimmilk Powder

1. Ascorbic acid

The results obtained for the content of total, free and masked sulfhydryl groups of skimmilk powder, containing increasing amounts of ascorbic acid added prior to irradiation, are presented in Fig. 10. The ascorbic acid additive has shown a protective effect on the skimmilk powder during irradiation at both applied doses, e.g. 0.4 and 1.6 Mrad. At all levels of ascorbic acid added there was a slight decrease of total sulfhydryl groups, which decrease was related to the amount of acid used. The protective effect of added ascorbic acid was most pronounced with additives up to 10 mM/100 g of powder; at higher amounts of added ascorbic acid, changes in the total -SH groups practically ceased, indicating that the protective role of the additive levelled off. For

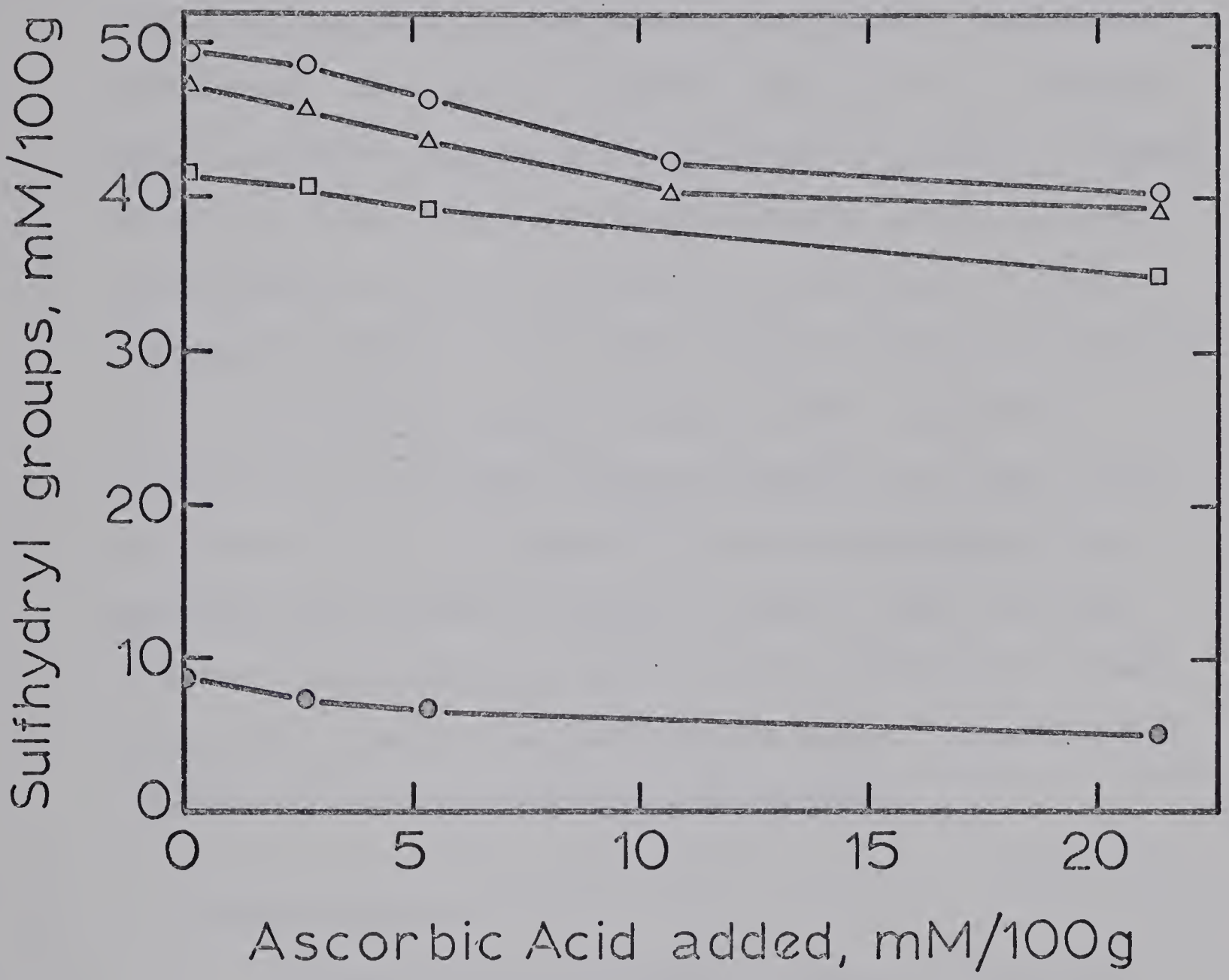


Fig. 10 Content of total, free and masked sulfhydryl groups found when laboratory skimmilk powder containing increasing amount of ascorbic acid was irradiated at 0.4 and 1.6 Mrad

Legend:

- △ *Total -SH at 0.4 Mrad irradiated skimmilk powder*
- ○ *Total -SH at 1.6 Mrad irradiated skimmilk powder*
- □ *Masked -SH at 1.6 Mrad irradiated skimmilk powder*
- ● *Free -SH at 1.6 Mrad irradiated skimmilk powder*

the first 5 mM of ascorbic acid added there was an approximate average decrease of -SH groups of 2 mM/100 g of skimmilk powder. This average decrease was obtained for both levels of irradiation dose applied. The content of available masked and free sulfhydryl groups also show a decrease at the dose level of 1.6 Mrad. As shown in Fig. 10, for the free (and masked) sulfhydryl groups the values obtained were 8.4 (41.2), 7.8 (40.7), 6.9 (39.4) and 5.1 (35.3) of -SH groups in mM/100 g of laboratory skimmilk powder in the presence of 2.7, 5.4, 11.9 and 23.8 mM of ascorbic acid, respectively. A slow decrease of the freely exposed sulfhydryl groups after irradiation from 8.4 to 5.1 mM suggested an additive protection of the available masked portion of sulfhydryl groups. Hence, the slow decrease in free sulfhydryl groups should be paralleled by a simultaneous slow increase of masked sulfhydryl groups. Instead of this, a decrease in content of both groups was obtained.

2. Ascorbyl palmitate

The data in Fig. 11 show that in common with ascorbic acid, ascorbyl palmitate was also effective in preventing rapid increases in the content of total sulfhydryl groups in irradiated laboratory skimmilk powders. These data show that in the presence of 2.3, 4.6, 9.3 and 18.6 mM of the additive there was a decrease from an initial value of 50 to 49.6, 47.1, 45.2, 40.7 mM of -SH per 100 g of powder, respectively, at an irradiation dose of 1.6 Mrad. At the lower dose level, 0.4 Mrad, the decreases were from

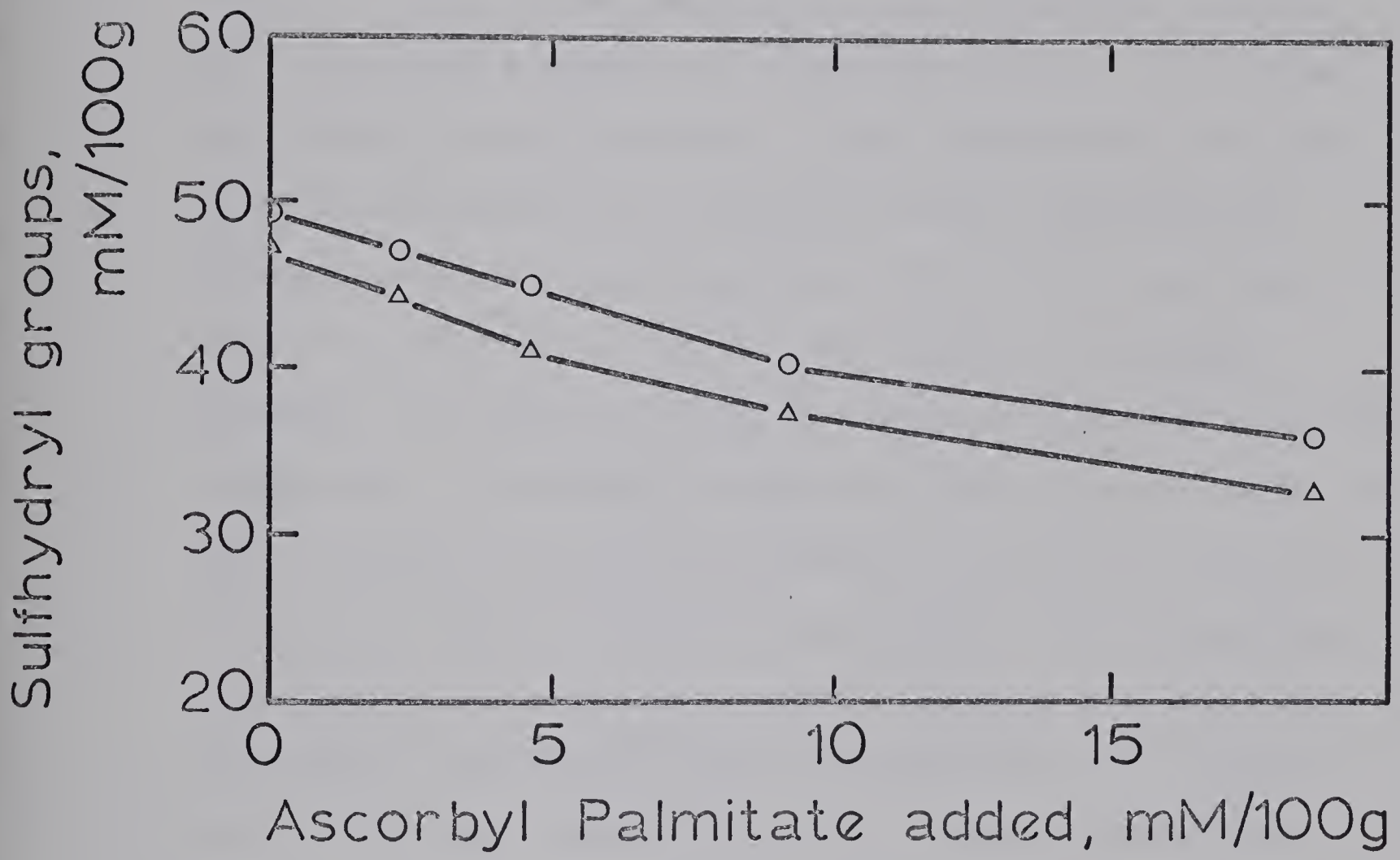


Fig.11 Total content of sulfhydryl groups found when laboratory skimmilk powder containing increasing amount of ascorbyl palmitate was irradiated at 0.4 and 1.6 Mrad

Legend: △ : *0.4 Mrad*
○ : *1.6 Mrad*

an initial value of 48.0, 44.4, 41.1, 37.8 and 32.9 mM of -SH groups per 100 g of powder. From these data, it might be concluded that ascorbyl palmitate was a more effective additive than ascorbic acid in preventing disulfide bond ruptures in proteins during irradiation. The concentrations added in these experiments were 2.7, 5.5, 11.9 and 23.8 mM/100 g of ascorbic acid and 2.3, 4.6, 9.3 and 18.6 mM/100 g ascorbyl palmitate. These concentrations were calculated from the quantities of additives actually incorporated in skimmilk powder on a percentage basis, e.g., 0.5, 1, 2 and 4 per cent of ascorbic acid, and 1, 2, 4 and 8 per cent of ascorbyl palmitate. The molecular weight of ascorbyl palmitate 414 is approximately twice (2.35x) that of ascorbic acid whose molecular weight is 176. Therefore, at an irradiation dose of 0.4 Mrad and in the presence of an increasing order of concentration of ascorbyl palmitate, the decreases in the per cent of total -SH groups, 5.5, 12.6, 19.7 and 29.9, in comparison to those of ascorbic acid, 2.4, 7.1, 14.2 and 16.5 per cent, respectively, does not reflect completely the superiority of ascorbyl palmitate as a protective additive. Hence, a comparison between the protective property of ascorbic acid and ascorbyl palmitate, as illustrated in Figs. 12 and 13, by a decrease in sulfhydryl groups to be valid on an accurate equimolar basis should involve for ascorbyl palmitate a multiplying factor of 1.35 for each decrease of sulfhydryl groups. Nevertheless, even neglecting this equimolar factor it is obvious that the ascorbyl palmitate was a more efficient protective additive. On the other hand, if

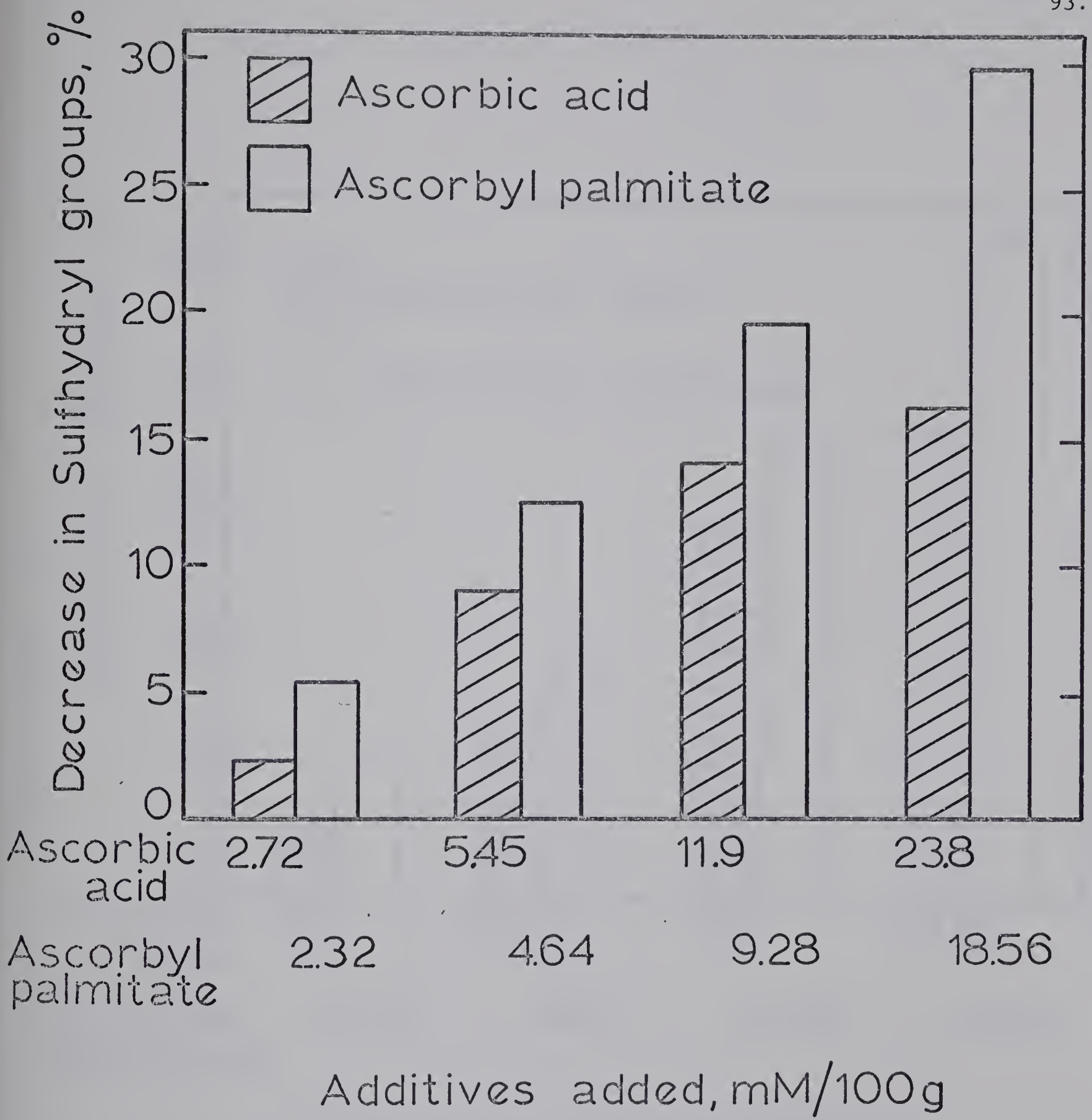


Fig. 12 Decreases in the total content of sulfhydryl groups when laboratory skimmilk powder was irradiated at 0.4 Mrad in the presence of additives

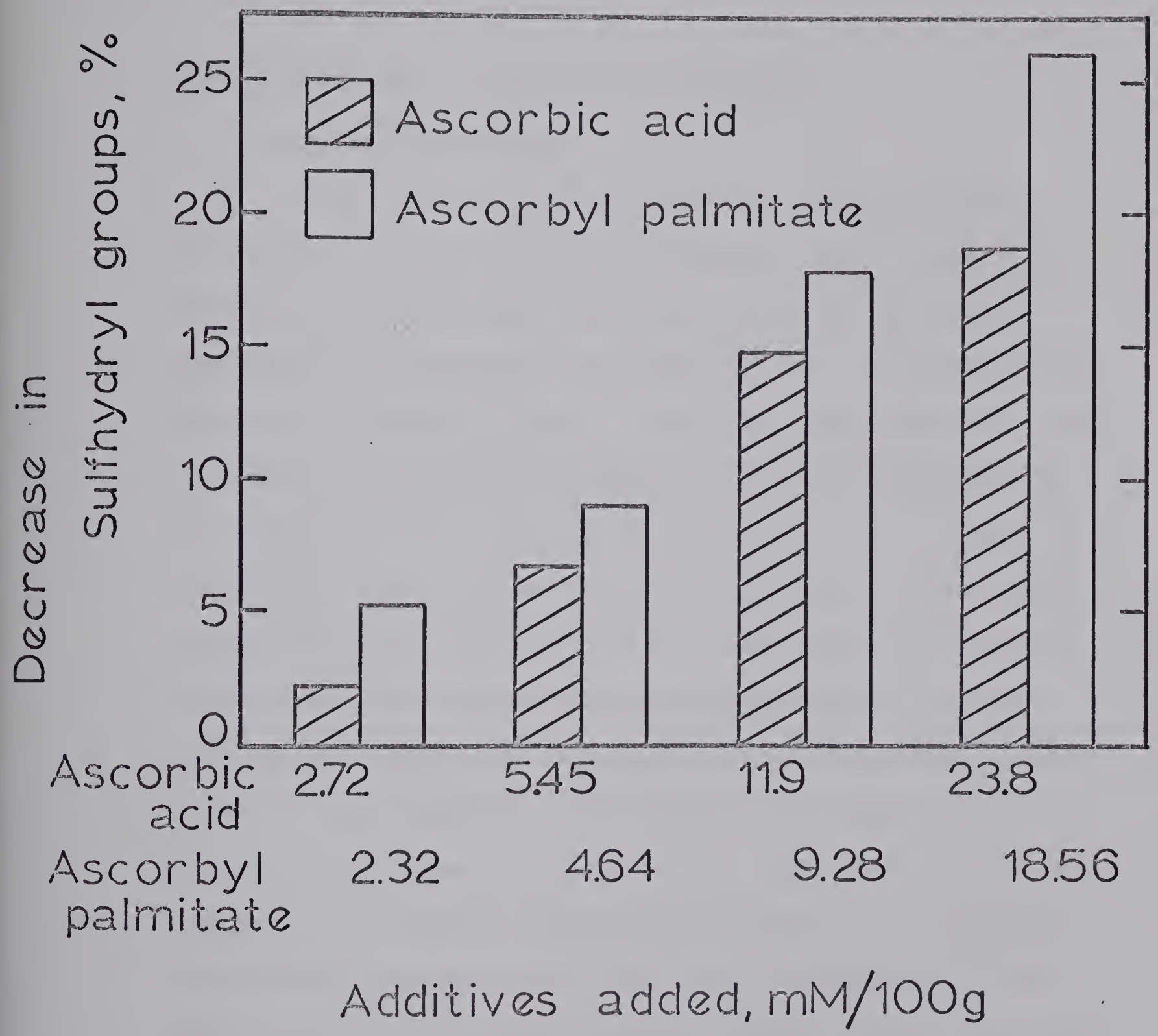


Fig. 13 Decreases in the total content of sulfhydryl groups when laboratory skimmilk powders were irradiated at 1.6 Mrad in the presence of additives

considered on an equimolar basis, the protective role of the molecule could not be ascribed solely to ascorbic acid but also to the palmitic moiety.

D. Degradation Products of Ascorbic Acid Additives

1. Thin layer chromatography

Equal volumes of C^{14}-ascorbic acid hydrazone extracts obtained from 0.4 and 1.6 Mrad irradiated and control, laboratory skimmilk powder containing 1 per cent ascorbic acid as a carrier were applied and separated on thin layer plates. The chromatograms obtained are presented in Fig. 14. All three samples applied formed an orange-red spot with an Rf value of 0.40 which corresponded to the derivative of the analytical grade ascorbic acid. In addition to this spot, the irradiated samples formed a second yellow-orange colored spot with an Rf value of 0.61. Since neither the standard ascorbic acid nor the non-irradiated samples revealed a similar spot this was assumed to be a degradation product of ascorbic acid produced during irradiation. The excess of the reagent used, 2,4-dinitrophenylhydrazine was located at Rf = 0.81, e.g., near the solvent front. To check the radioactivity distribution on the plate a radioactivity scanning was performed. For this purpose 40 µl of each of the C^{14}-ascorbic acid hydrazone extracts obtained from irradiated and non-irradiated skimmilk powders were applied as bands along the start line of the plates (5 x 20 cm). The Rf values, as well as the colors of each band obtained, corresponded to those

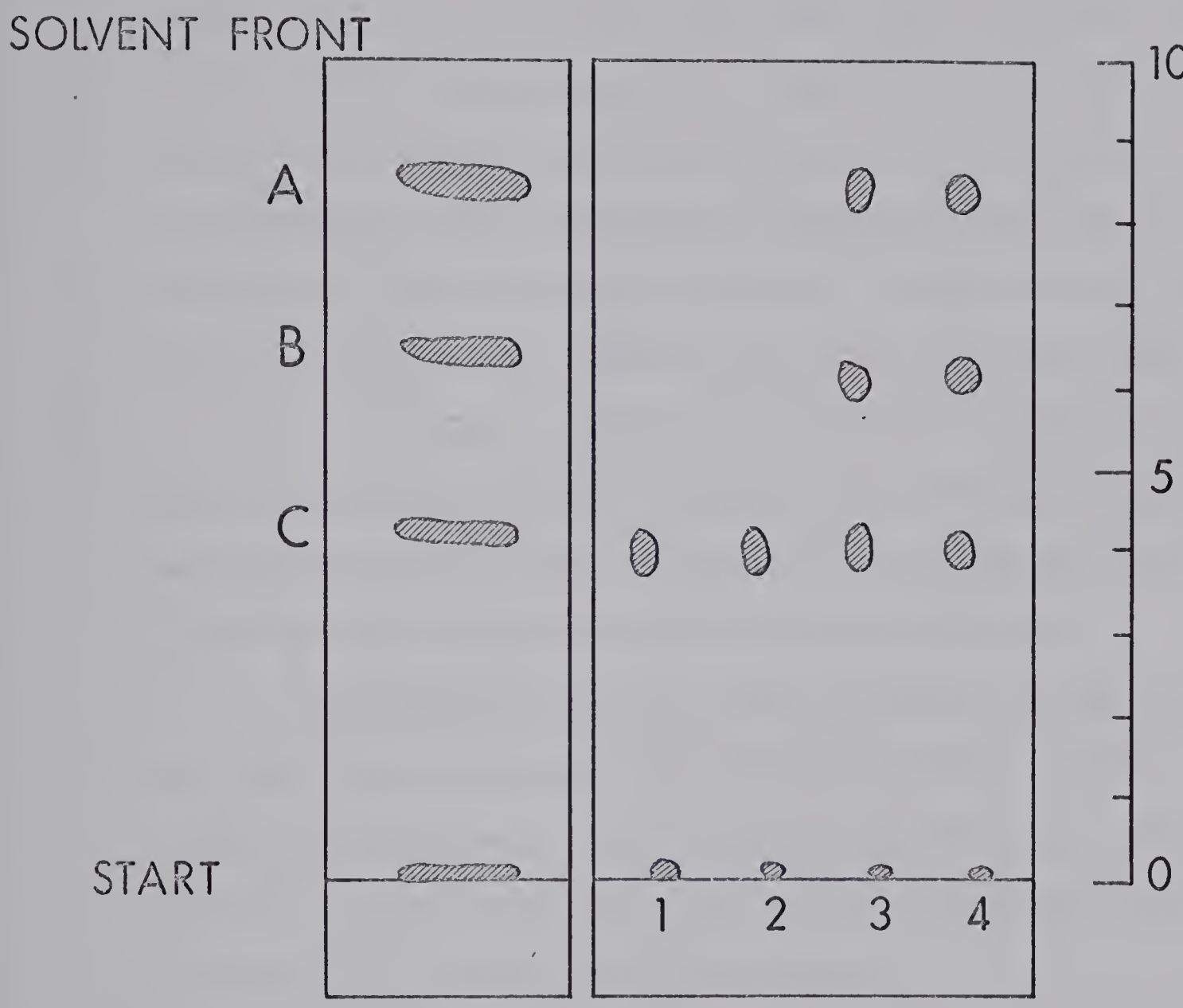


Fig. 14 Thin-layer chromatograms of ascorbic acid additive and its degradation products isolated from irradiated and non-irradiated laboratory prepared skimmilk powder

Plates: 250 µ Silica gel G

Solvent system: Diethylether, Ethyl acetate, Acetic acid, 75:25:4 (v/v)
Development: 20 min at room temperature

1. pure ascorbic acid; 2. control; 3. 0.4 Mrad; 4. 1.6 Mrad; A. Excess of 2.4 DNPH (yellow), Rf = 0.84; B. Degradation product (yellow-orange), Rf = 0.61; C. ascorbic acid hydrazone (orange-red), Rf = 0.40

obtained previously for spot separation. The results of scanning are presented in Fig. 15. The control sample area and those of the samples irradiated as 0.4 and 1.6 Mrad were designated as I, II and III, respectively. As can be seen the control sample produced a sharp narrow triplet, designated as A, in which two smaller peaks were shouldering the major centrally located peak. The scanning of the two irradiated samples revealed two additional bands from both sides of the major peak designated as B and C. In spite of the increased dose of irradiation the radioactivity of the additional bands B and C did not increase, but the central main peak decreased in activity. This finding indicates that a portion of the labelled ascorbic acid was degraded and also that a portion of the degradation products formed was not recovered as 2,4-dinitrophenylhydrazone.

The radioactivity of the separated bands was also estimated by scintillation counting, which results are shown in Table 7. The results indicated that in band C as the irradiation dose was increased the C^{14}- activity decreased, while in band B the radioactivity increase followed the increase of irradiation dose.

2. Gas liquid chromatography

Additionally to assess the protecting role of ascorbic acid during irradiation the volatiles produced in its presence were further analyzed. To obtain the volatiles laboratory skimmilk powder containing 1 per cent ascorbic acid was irradiated at a maximum dose of 1.6 Mrad. The volatiles produced were then extracted in ethyl ether and injected straight into the GLC column. The chromatogram obtained is shown in Fig. 16. There were altogether 34 peaks identified ranging in retention times from 0 to 180 mins. The most pro-

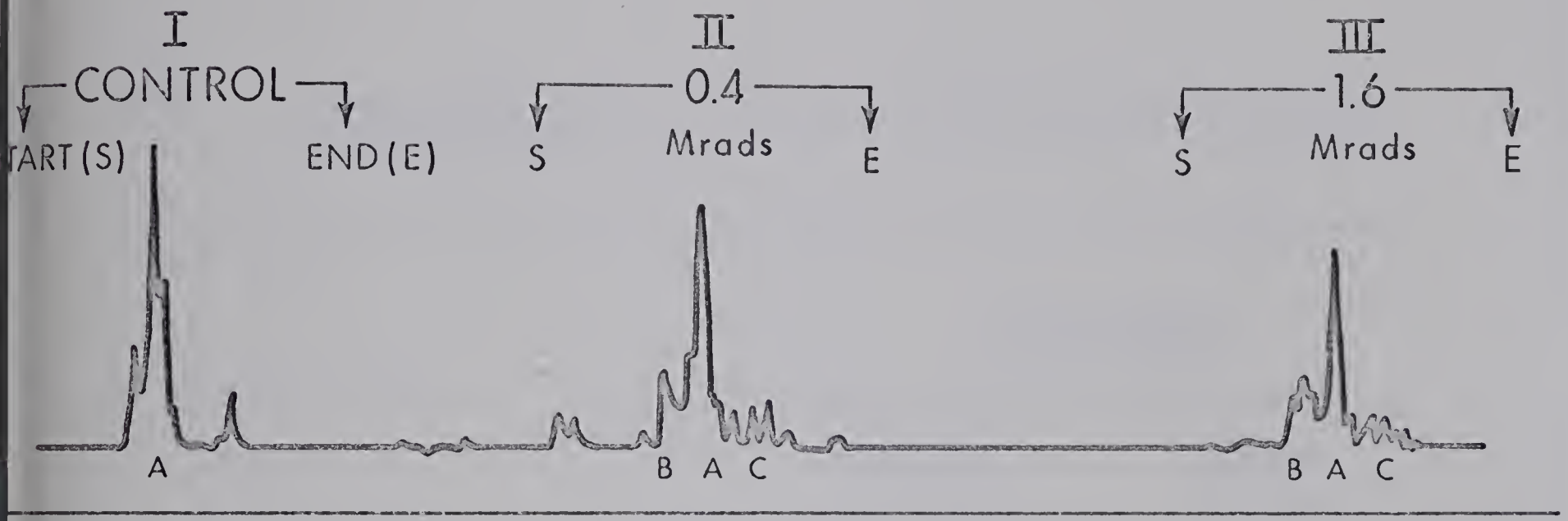


Fig. 15. *Thin layer radiochromatography scanning of ascorbic acid additive and its degradation products isolated from laboratory prepared and irradiated skimmilk powder*

Scanning unit: *Nuclear Chicago*
TL plate conveyor system, Model 1006

Area I : *Control sample*
Area II : *0.4 Mrad irradiated sample*
Area III: *1.6 Mrad irradiated sample*

Band A = ascorbic acid
Band B and C = degradation products

Table 7 - Recovered C^{14}-ascorbic acid from non-irradiated and irradiated skimmilk powder

Irradiation dose	Radioactivity counts /min *	
Mrad	Band 1 Rf = 0.40	Band 2 Rf = 0.61
Non-irradiated	1545	85
0.4	1474	103
1.6	1379	192

* Uncorrected for quenching effect due to silicic acid

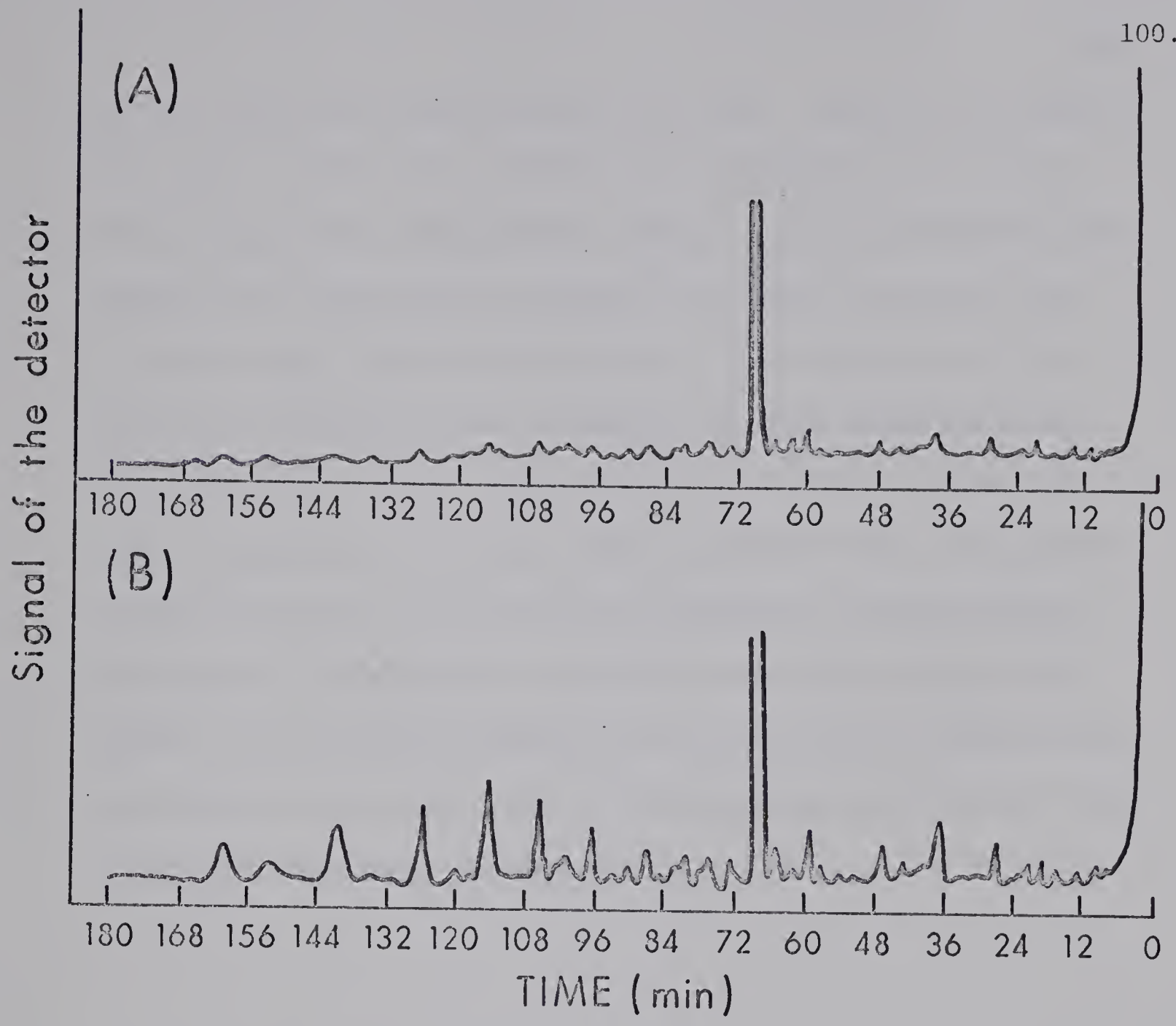


Fig. 16 *The GLC chromatograms of diethyl ether extracts of degradation products of ascorbic acid additive from non-irradiated (A) and irradiated (B) skimmilk powders.*

Gas Chromatograph: *Bendix Model 2500*

Liquid phase: *20 per cent of Carbowax 20 M on 80 - 100 mesh acid-washed Chromosorb P*

Carrier gas flow rate: *60 ml/min of helium*

Sensitivity: *Input attenuation,* x 100; *Recorder attenuation,* x 5; *Suppression range,* x 100

Temperature programming: *At* $110^{\circ}C$ *for 3 min and then programmed to* $215^{\circ}C$ *at a rate of* 1°*/min*

nounced peak had a retention time of 67 mins. Based on the retention times recorded, the volatiles from irradiated and non-irradiated samples were qualitatively similar. All the retention times obtained were subsequently compared to the known retention times of ascorbic acid degradation products, such as furfural and its derivatives, 2-acetyl-furan, 2-furfuryl alcohol, gamma-butyrolactone, furil and 2-furoic acids. However, only four, 2-furfuryl alcohol, gamma-butyrolactone, 2-furoic acid and furil corresponded to those found in irradiated and non-irradiated skimmilk powders. Nevertheless, the protective role of ascorbic acid was again confirmed since the sulfur-containing volatiles with low boiling points and with retention times from 1 to 40 mins were simultaneously detected only in negligible amounts.

V. DISCUSSION

A. The Effects of Irradiation on the Sulfhydryl Groups in Skimmilk Powder

The rupture of the disulfide bonds in milk proteins by irradiation results in a release of free sulfhydryl groups. Dependant on the sterical position of the disulfide bond this rupture might bring about an internally located, hindered or masked -SH group within the protein or an exposed, externally located -SH group. In addition to such sterical possibilities, probably an unfolding process also occurs which might bring about more of the internal -SH groups to the surface to become an easily accessible fraction of free -SH group. As our results have shown the amounts of free and masked -SH groups found in skimmilk powder after irradiation might reflect both processes to occur in milk proteins. As presented in Tables 3 and 4 and Fig. 6, the amounts of free and masked -SH groups formed in skimmilk powder after irradiation were also related to the irradiation doses applied. All these results were expected and might serve as a further confirmation of the results reported in previous studies (McArdle and Desrosier, 1955, Kraybill et al. 1960).

The ascorbic acid and ascorbyl palmitate additives were used as radical scavengers. The use of ascorbic acid labelled with C^{14} in position C_1 has shown that a portion of the additive degraded during irradiation of skimmilk powder which could not be

recovered as carbonyls. The use of uniformly labelled ascorbic acid might give an unequivocal answer for degradation steps involved but such an ascorbic acid is not yet available commercially. Hence, the mechanism of their protective role during irradiation was not clarified. Nevertheless, the results obtained for the change of free, masked and total -SH groups confirmed their protective role.

B. The Effect of Irradiation on Volatiles

1. High boiling point volatiles

As previously indicated the typical off-flavor of dried milk powder is a composite of two distinct flavor sensations, one of which arises from the oxidation of lipids and the other from the non-enzymatic heat induced browning reaction.

The skimmilk powder used in the present study contained 0.6 - 0.7 per cent fat as determined by a method based on the ether extraction procedure. This amount of residual fat may exist in milk powder as globules protected by an adsorbed protein-phospholipid layer and partly as free or non-globular fat absorbed by the dried milk particles. As reported by Lindquist and Brunner (1962) the free fat of spray dried whole milk powder contains slightly more neutral glycerides, saturated fats of C_{10} - C_{18} and less mono and diglycerides, phospholipids and unsaturated fats, than the total milk fat. This pool of free fat, especially that adsorbed on particle surfaces, as claimed by Greenbank and Pallanch (1962),

is more rapidly oxidized than the globular fat.

The laboratory skimmilk powder used in the present study was prepared by freeze-drying and would be expected to contain a much smaller content of free fat than the commercially dried skimmilk, which is subjected to much greater milk fat-globule destabilizing influences. Nevertheless, the volatiles recovered from both skimmilk powders had essentially the same composition, e.g. mostly straight chain aldehydes and ketones and straight chain alkanoic acids. The procedure applied in the present study for recovering volatiles involved a simulated steam distillation technique. The reconstituted milk was heated at 40°C with a vacuum of 4 mm Hg, under which condition the water evaporated along with other volatiles.

The ice-water trap fraction collected was extracted with diethyl ether and without further separation into carbonyl- and acid-containing fractions was subjected to identification by GLC. The distillate trapped in liquid nitrogen was treated with mercuric chloride to precipitate the sulfur-containing volatiles. From this distillate the clear supernatant contained most of the volatiles having a retention time of 3.5 mins or less. As found in a parallel GLC study these volatiles contained from C_4 to C_6 aldehydes and furfural, ketones, 2,3-butadione and 2-pentanone and alcohols such as methanol, ethanol, n- and iso-propanols, and n-, iso- and sec-butanols. These low b.p. volatiles were found by Bassette and Keeney (1960) to be normal constituents of the volatiles of skimmilk powder.

The GLC chromatographs (Figs. 7 and 16) represent the medium and high boiling point volatiles since the traces of low boiling point volatiles, under the conditions applied would be removed with peak No. 1, which represents the ethyl ether solvent. Theoretically, the medium and high b.p. volatiles should consist of aldehydes from C_7 - C_{12}, ketones from 2-hexanone to 2-decanone and alkanoic acids, consisting of acetic, iso- and n-butyric acids, valeric, caproic, caprylic, capric and lauric acids. In addition to these normal constituents expected to be found in these volatiles, the GLC separation presented in Fig. 16 should contain the ascorbic acid degradation products, such as, furfural, 2-furoic acid, gamma-butyrolactone, furil and 2-furfuryl alcohol.

Actually, based on retention times, only n-heptanal (1) and benzaldehyde were detected with certainty. The other aldehydes, such as octanal, nonanal, decanal, dodecanal and myristic aldehyde, usually present in skimmilk powder, according to Bassette and Keeney (1960), were not confirmed. From the ketone fraction, 2-octanone (1) and 2-nonanone were confirmed. Earlier reports confirmed the presence in skimmilk powder of acetone, 2-butanone, 2-pentanone and 2,3-butadione (Bassette and Keeney 1960). Muck et al. (1963) reported the presence in aged evaporated milk of 2-pentanone, 2-heptanone,

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(1) See appendix Table 1.

2-nonanone, 2-undecanone and 2,3-decanone. In fresh and stored casein volatiles Ramshaw and Dunstone (1969) reported the presence of the following ketones, 2,3-butadione, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone and 2-dodecanone. In the present research organic acids, acetic(1), isobutyric, caproic, heptanoic, caprylic were confirmed on the basis of their retention time. Caprylic acid was present in a concentration higher than all of the volatiles detected. In a study of the volatiles of fresh and stored casein Ramshaw and Dunstone reported that the organic acids, valeric, caprylic, and capric were present in the amounts of 43, 35 and 14 per cent, respectively. Previously, in skimmilk powder Kawanishi and Saito (1966) reported the presence of n-butyric and n-caproic acids as volatile constituents.

In the irradiation experiments in which ascorbic acid was used as a scavenger additive the volatiles recovered from the fragmentation of the ascorbic acid were 2-furfuryl alcohol(2), gamma-butyrolactone, 2-furoic acid and furil. Furfural, a common degradation product of ascorbic acid, was not recovered by the technique used because of its low retention time of 2.4. In an analytical study of the degradation products formed when ascorbic acid was heated in an aqueous solution Tatum et al. (1969) reported the isolation of 10 furan-type compounds, 2 lactones, 3 acids and 3-hydroxy-2-pyrone. In separating these compounds on Carbowax

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(1) See appendix Table 1.
(2) See appendix Table 2.

20 M the retention times obtained by these investigators were somewhat different from those obtained in the present study, since the column they used in their separation was 9 ft long and the temperature programming was different.

In addition to the volatiles produced by the degradation of added ascorbic acid other volatiles were also obtained that were similar to those presented in Fig. 7. The only exceptions were the aldehydes that could not be confirmed with certainty in this GLC separation. As reported above, the organic acids were present in large amounts. The acids were butyric, n-valeric, caproic, heptanoic, caprylic and nonanoic. The caprylic acid was a major volatile and its content was increased by irradiation of the powder. No published information on the volatiles produced by the irradiation of ascorbic acid fortified skimmilk powder is available.

Attention is drawn to the increased number of ascorbic acid volatiles obtained in this research. Contrary to the procedure in a previous experiment (Fig. 7), in which the volatiles were obtained by distillation, the volatiles in these experiments were extracted with ether, consequently the extract contained the volatiles isolated by steam or vacuum distillation plus the compounds which are not steam volatile but easily extractable in ether. Ramshaw and Dunstone (1969) reported that compounds non-volatile in steam vapour, consisting mostly of aromatic compounds such as benzyl alcohol, benzthiazole, cresol, phenol and o-aminoacetophenone to be present in both fresh and stored casein. The latter compound

was also confirmed to be present in skimmilk powder by Parks et al. (1964). In the present investigation there was no separation of the sulfurous volatiles by precipitation. Consequently, there should be at least 50 volatile compounds detected rather than the 34 volatiles represented by the well distinguished GLC peaks in Fig. 16. This discrepancy between the expected and actual finding may be explained by the fact that many of the volatile compounds have the same retention times under the separation conditions used, hence, one peak in the GLC chromatogram may represent several volatile compounds. Supporting this assumption are the retention times obtained for butyric acid and heptanal (R.T. 3.5), as well as for benzaldehyde and 2-nonanone (R.T. 5.8 - 6.0).

2. Sulfur-containing volatiles

The sulfur-containing volatiles present in skimmilk powder in considerable amounts before irradiation were those with retention times of 3.1, 17.5, 21.2, 27.8 and 35.6 mins. After irradiation there was a substantial increase in volatiles with retention times of 4.9, 6.7, 13.3, 23.4 and 39.5 mins. By the application of co-chromatography technique some of these volatiles were identified as ethyl-, n-propyl-, n-butyl, sec-butyl- and allyl mercaptans and diallyl sulfide. Undoubtedly, the -SH free radicals for these volatiles was provided from methionine and/or cysteine, cystine amino acids as precursors, while the hydrocarbon moiety could arise from free radicals such as methyl-, ethyl-, propyl-, butyl-, iso-butyl- and iso-amyl-. The constituents of milk powder might

supply a choice of hydrocarbons from polypeptides, amino acids and short chain fatty acids through reactions involving decarboxylation, deamination, homolytic carbon chain ruptures, etc.

No data exists in the literature that supports unequivocally the mechanism for the formation of sulfur-containing volatiles, as suggested above, and neither was an elucidation of the chemical reactions involved an objective of this study. It has, however, been shown that the irradiation of skimmilk powder results in the formation of a considerable number of sulfur-containing volatiles that undoubtedly contribute to the off-flavor detected when irradiated skimmilk powder is recombined with water.

APPENDIX

TABLE 1 - Retention times of the volatiles from commercial skimmilk powder recovered in ice-water trap.

Column Carbowax 20M temperature programming 100 - 200° at 8°/min.

SAMPLE			
Control	Irradiated 1.6 Mrad	Relative Abundance*	Compounds** which under the same conditions of separation have equal retention time
	3.5	++	acetic acid
	4.7	+	heptaldehyde
	6.7	++	iso-butyric acid
	7.4	++	benzaldehyde
	8.8	+	valeric acid
	9.0	++	
9.3	9.3	+	2-nonanone
	10.0	+	
10.4	10.4	+	
	10.6	+	
11.3	11.3	++	caproic acid
	11.5	+	
11.9	11.8	+	
12.5	12.4	+	heptanoic acid
12.7	12.7	++	
13.4	13.4	+++	caprylic acid
14.5	14.5	+	
15.4	15.3	+	
15.8	15.8	++	
16.2	16.2	++	
17.2	17.1	++	
	17.3	+	
	18.0	+	
	18.3	+	
	19.0	+	
20.2	20.2	++	
	20.8	+	
21.8	21.8	+	
25.8	25.7	++	
31.4	31.3	+++	

* + low
++ medium
+++ high

** The analytical grade compounds were obtained from Eastman Kodak Co., Tennessee, and Aldrich Chem. Co., Milwaukee, Wisconsin.

APPENDIX

TABLE 2 - The retention times of the volatiles recovered from laboratory skimmilk powder irradiated at 1.6 Mrad in presence of ascorbic acid additive.

Column Carbowax 20M temperature programming 110 - 215° at 1°/min.

SAMPLE			
Control	Irradiated	Relative Abundance*	Compounds** which under the same conditions of separation have equal retention time
3.6	3.6	+	n-butyric acid
5.2	5.2	+	benzaldehyde
7.3	7.3	+	n-valeric acid
8.5	8.5	+	2-furfuryl alcohol
9.7	9.7	+	gamma-butyrolactone
13.0	13.0	++	
16.1	16.1	+	caproic acid
19.0	19.0	++	
22.0	22.0	++	
23.4	23.4		heptanoic acid
27.6	27.6	++	
37.0	37.0	++	caprylic acid
43.0	43.0		
46.3	46.3	++	nonanoic acid
56.7	56.7	+	2-furoic acid
58.8	58.8	++	
61.0	61.0	+	
64.5	64.5	+	
67.5	67.4	+++	
71.0	71.0	++	
76.0	76.0	++	furil
80.5	80.5	+	
82.0	82.0	+	
86.4	86.4	++	
91.3	91.3	+	
92.0	92.0	+	
93.0	93.0	+	
97.0	97.0	++	
106.0	106.0	++	
115.0	115.0	++	
118.5	118.5		
126.0	126.0	++	
153.5	153.5	++	
162.0	162.0	++	

* + low
++ medium
+++ high

** The analytical grade compounds were obtained from Eastman Kodak Co., Tennessee, and Aldrich Chem. Co., Milwaukee, Wisconsin.

APPENDIX

TABLE 3 - Retention times of some low boiling point volatiles on Carbowax 20M column applying two different temperature programmings*.

COMPOUNDS**	100 - 200° at 8°/min	100 - 215° at 1°/min
Alcohols:		
Methanol	0.7	0.3
Ethanol	0.9	0.4
Aldehydes:		
n-Hexanal	2.6	1.8
n-Heptanal	5.0	3.3
Furfural	-	2.4
Ketones:		
2,3-Butadione	1.3	0.6
Acetone	0.8	0.3
2-Pentanone	1.4	1.5
2-Heptanone	5.8	2.7
Acids:		
Acetic	3.9	0.6
Iso-Butyric	6.4	2.4

* Since the retention times of these compounds are less than 3.6 they were not separated and reported as volatiles in Tables 1 and 2.

** The analytical-grade compounds were obtained from Eastman Kodak Co., Tennessee, and Aldrich Chem. Co., Wisconsin.

VII. REFERENCES

Alexander, P., M. Fox, K. A. Stacey and D. Rosen. 1956. Comparison of some direct and indirect effects of ionizing radiations in protein. Nature. 178 : 846-9.

Ampulski, R. S., V. E. Ayers and S. A. Morell. 1969. Determination of the reactive sulfhydryl groups in heme proteins with 4,4'-dipyridinedisulfide. Anal. Biochem. 32 : 163-9.

Barr, N. F. and C. G. King. 1956. The gamma ray oxidation of ascorbic acid and ferrous ions. J. Am. Chem. Soc. 78 : 303-5.

Barratt, B. 1967. Gamma irradiation. Can. Food Ind. 38 : 19.

Barron, E. S. G. and V. Flood. 1950. Studies on the mechanism of action of ionizing radiations. VI. The oxidation of thiols by ionizing radiations. J. Gen. Physiol. 33 : 229-41.

Bassette, R. and M. Keeney. 1960. Identification of some volatile carbonyl compounds from nonfat dry milk. J. Dairy Sci. 43 : 1744-50.

Beeby, R. 1964. The presence of sulfhydryl groups κ-casein. Biochim. Biophys. Acta. 82 : 418-9.

Benesch, R. E., H. A. Lardy and R. Benesch. 1955. The sulfhydryl groups of crystalline proteins. J. Biol. Chem. 216 : 663-76.

Bierman, G. W., B. E. Proctor and S. A. Goldblith. 1956. Radiation preservation of milk and milk products. II. Off-flavors in milk and cream induced by ionizing radiation as judged by organoleptic tests. J. Dairy Sci. 39 : 379-90.

Bloksma, A. H. 1959. The amperometric titration of thiol groups in flour and gluten. Cereal Chem. 36 : 357-68.

Boyd, E. N. and I. A. Gould. 1957. Volatile and non-volatile sulfhydryl content of heated milk and milk products. J. Dairy Sci. 40 : 1294-307.

Boyer, P. D. 1954. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. J. Am. Chem. Soc. 76 : 4331-7.

Braams, R. 1963. A mechanism for the direct action of ionizing radiations. Nature. 200 : 752-4.

Brown, W. D. 1960. Reduction of protein disulfide bonds by sodium borohydride. Biochim. Biophys. Acta. 44 : 365-7.

Brownell, L. E., C. A. Lawrence and J. T. Graikoski. 1953. Sterilizing foods by cold gamma rays. Refrig. Eng. 61 : 55-7.

Burton, H. 1958. The amperometric titration of sulfhydryl groups at a rotating platinum electrode. Biochim. Biophys. Acta. 29 : 193-201.

Butterworth, P. H. W., H. Baum and J. W. Porter. 1967. A modification of the Ellman procedure for the estimation of protein sulfhydryl groups. Arch. Biochem. Biophys. 118 : 716-23.

Copeland, E. S., T. Sanner and A. Pihl. 1968. Role of intermolecular reactions in the formation of secondary radicals in proteins irradiated in the dry state. Radiation Research. 35 : 437-50.

Day, E. A., D. A. Forss, and S. Patton. 1957. Flavor and odor defects of gamma-irradiated skimmilk. I. Preliminary observations on the role of volatile carbonyl compounds. II. Identification of volatile components by gas chromatography and mass spectrometry. J. Dairy Sci. 40 : 922-41.

Dluzewski, M. and E. Pijanowski. 1964. Effect of gamma-irradiation on the coliform bacteria in raw milk. Nuclear Sci. Abs. 19 : 13825.

Dunn, C. G., W. L. Campbell, H. Fram and A. Hutchins. 1948. Biological and photochemical effects of high energy electrostatically produced roentgen rays and cathode rays. J. Appl. Phys. 19 : 605-16.

Ellman, G. L. 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82 : 70-7.

Freeman, B. M. 1959. Bacteriological investigation of liquid milk irradiated in gamma source. Intern. J. Appl. Rad. Isotop. 6 : 160-3.

Gaden, E. L. Jr., E. J. Henley and V. P. Collins. 1951. Preservation of milk by radiation. Food Technol. 5 : 506-9.

Goldblith, S. A. 1970. Radiation preservation of food - current status. J. Food Technol. 5 : 103-10.

Goldblith, S. A. and B. E. Proctor. 1954. Radiation sterilization. VI. Relative merits of cathode rays and gamma radiations. Nucleonics. 12 : 32-5.

Goldblith, S. A. and B. E. Proctor. 1956. Radiation preservation of milk and milk products. I. Background and problems. J. Dairy Sci. 39 : 374-8.

Glaser, C. B., H. Maeda and J. Meinenhofer. 1970. Chromatographic detection of thiols, disulfides and thioesters with 5,5'-dithio-bis-(nitrobenzoic acid). J. Chromatog. 50 : 151-4.

Gordy, W., W. B. Ard and H. Shields. 1955. Microwave spectroscopy of biological substances. I. Paramagnetic resonance of x -irradiated amino acids and proteins. Proc. Natl. Acad. Sci. U.S. 41 : 983-96.

Grassetti, D. R. and J. F. Murray Jr. 1967. Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiopyridine. Arch. Biochem. Biophys. 119 : 41-9.

Green, G. R. and M. J. Pallanch. 1962. The progress of oxidation in the milk powder granule. XVI. Intern. Dairy Cong. B1002-8.

Grim, A. C. and S. A. Goldblith. 1965. The effect of ionizing radiation on the flavor of whole-egg magma. Food Technol. 19 : 1594-96.

Harland, H. A. and U. S. Ashworth. 1945. The use of thiamine disulfide for the estimation of reducing substances in processed milk. J. Dairy Sci. 28 : 15-23.

Harper, K. A., A. D. Morton and E. J. Rolfe. 1969. The phenolic compounds of blackcurrant juice and their protective effect on ascorbic acid oxidation and its inhibition by flavonoids. J. Food Technol. 4 : 255-67.

Henricksen, T., T. Sanner and A. Pihl. 1963. Transfer of radiation induced unpaired spins from proteins to sulfur compounds. Radiation Research. 18 : 163-76.

Hill, R. D. 1964. The cysteine content of casein micelle. J. Dairy Sci. 31 : 285-90.

Hutton, J. T. and S. Patton. 1952. The origin of sulfhydryl groups in milk proteins and their contributions to "cooked" flavor. J. Dairy Sci. 35 : 699-705.

Kalab, M. 1970. Factors affecting the Ellman determination of sulfhydryl groups in skimmilk powder and gels. J. Dairy Sci. 53 : 711-8.

Kawanishi, G. and K. Saito. 1966. Flavour components in dried whole milk and skimmilk. Dairy Sci. Abs. 29 : 2852.

Koch, A. L. 1958. The absence of radiation-induced disulfide interchanges. In: Symposium on Information Theory in Biology by Yockey, H. P. Pergamon Press. New York. p. 283-5.

Kraybill, H. F., M. S. Read, R. S. Harding and T. E. Friedemann. 1960. Biochemical alteration of milk proteins by gamma and ultraviolet irradiation. Food Res. 25 : 372-8.

Kumta, U. S., F. Shimazu and A. L. Tappel. 1962. Decrease of radiation damage to proteins by sulfhydryl protectors. Radiation Research. 16 : 679-85.

Kumta, U. S. and A. L. Tappel. 1961. Radiation damage to proteins. Nature. 191 : 1304-5.

Kung, H., E. L. Gaden and C. G. King. 1953. Vitamins and enzymes in milk. Effect of gamma radiation on activity. J. Agr. Food Chem. 1 : 142-4.

Lagercrantz, C. 1964. Free radicals in the auto-oxidation of ascorbic acid. Acta. Chem. Scand. 18 : 562.

Larson, B. L. and R. Jenness. 1950. The determination of protein sulfhydryl groups with iodine and o-iodosobenzoate by an amperometric titration. J. Dairy Sci. 33 : 890-5.

Licciardello, J. J. 1964. Effect of temperature on radiosensitivity of Salmonella typhimurium. J. Food Sci. 29 : 469-74.

Lindquist, K., and J. R. Brunner. 1962. Composition of free fat of spray-dried whole milk. J. Dairy Sci. 45 : 661.

Littman, F. E., E. M. Carr and J. K. Clauss. 1957. Protection of sulfhydryl groups against ionizing radiation. Science. 125 : 737-8.

Lyster, R. L. J. 1964. The free and masked sulfhydryl groups of heated milk and milk powder and a new method for their determination. J. Dairy Res. 31 : 41-51.

Matsumoto, H., and J. Hlynka. 1959. Some aspects of the sulfhydryl-disulfide system in flour and dough. Cereal Chem. 36 : 513-21.

McArdle, F. J. and N. W. Desrosier. 1955. Influences of ionizing radiations on the protein components of selected foods. Food Technol. 9 : 527-321.

Mecham, D. K. and C. Knapp. 1966. The sulfhydryl contents of doughs mixed under nitrogen. Cereal Chem. 43 : 226-36.

Muck, G. A., J. Tobias and R. McL. Whitney. 1963. Flavour of evaporated milk. I. Identification of some compounds obtained by the petroleum ether solvent partitioning technique from aged evaporated milk. J. Dairy Sci. 46 : 774-9.

Neurath, H. and K. Bailey. 1954. The Proteins Vol. II Part A. Academic Press Inc., New York. p.395-411.

O'Meara, J. P. 1952. Radiation chemistry and sterilization of biological materials by ionizing radiation. Nucleonics. 10 : 19-23.

Parks, O. W., D. P. Schwartz and M. Keeney. 1964. Identification of o-aminoacetophenone as a flavour compound in state dry milk. Nature. 202 : 185-7.

Patten, F. and W. Gordy. 1961. Temperature effects on the formation of free radicals in the amino acids. Radiation Research. 14 : 573-89.

Patten, R. A. and W. Gordy. 1964. Further studies of radiation effects on the proteins and their constituents. Radiation Research. 22 : 29-44.

Patton, S. and D. V. Josephson. 1949. Observations on the application of the nitroprusside test to heated milk. J. Dairy Sci. 32 : 398-405.

Proctor, B. E. and S. A. Goldblith. 1951. Food Processing with ionizing radiations. Food Technol. 5 : 376-80.

Proctor, B. E., S. A. Goldblith, C. G. Bates and O. A. Hammerle. 1952. Biochemical changes in foods and tissues sterilized by ionizing radiations. Food Technol. 6 : 237-42.

Proctor, B. E. and J. P. O'Meara. 1951. Effect of high voltage cathode rays on ascorbic acid. Ind. Eng. Chem. 43 : 718-21.

Ramshaw, E. H. and E. A. Dunstone. 1969. Volatile compounds associated with the off-flavour in stored casein. J. Dairy Res. 36 : 215-23.

Rao, B. S. N. 1962. Radiolysis of ascorbic acid in aqueous solution by gamma radiation. Radiation Research. 5 : 683-93.

Read, M. S., H. F. Kraybill and N. F. Wih. 1958. Short term rat feeding studies with gamma-irradiated food. J. Nutrition. 65 : 39-51.

Sasago, K., H. K. Wilson and E. O. Herreid. 1963. Determination of sulfhydryl and disulfide groups in milk by the p-chloromercuribenzoate-dithizone method. J. Dairy Sci. 46 : 1348-51.

Scanlan, R. A. and R. C. Lindsay. 1968. Observations on the low temperature irradiation of milk. J. Dairy Sci. 51 : 1967-8.

Sedlak, J. and R. H. Lindsay. 1968. Estimation of total protein-bound and non-protein sulfhydryl groups in tissues with Ellman's reagent. Anal. Biochem. 25 : 192-205.

Sharpless, H. E., A. E. Blair and C. R. Maxwell. 1955. The effect of ionizing radiations on amino acids. II. The effect of x-rays on the aqueous solutions of alanine. Radiation Research. 2 : 135-44.

Sokol, H. A., D. K. Mecham and J. W. Pence. 1959. Further studies on the determination of sulfhydryl groups in wheat flour. Cereal Chem. 36 : 127-33.

Sullivan, R. A., R. A. Hollis and E. K. Stanton. 1957. Sedimentation of milk proteins from heated milk. J. Dairy Sci. 40 : 830-3.

Suzuki, M., T. L. Coombs and B. L. Vallee. 1969. Determination of protein thiol groups by atomic absorption spectrometry. Anal. Biochem. 32 : 106-17.

Tatum, J. H., P. E. Shaw and R. E. Berry. 1969. Degradation products from ascorbic acid. J. Agr. Food Chem. 17 : 38-40.

Thornley, M. J. 1963. Radiation control of Salmonella in food and feed products. Intern. Atomic Agency, Vienna. p.81.

Townley, R. C. and I. A. Gould. 1943. A quantitative study of the heat labile sulfides of milk. I. Method of determination and influence of temperature and time. J. Dairy Sci. 26 : 689-703.

Tsen, C. C. and W. Bushuk. 1968. Reactive and total sulfhydryl and disulfide contents of flavors of different mixing properties. Cereal Chem. 45 : 58-62.

Tsugo, T. and T. Hayashi. 1961. Effect of irradiation by ionizing radiation on milk enzymes. I. Preliminary experiments and irradiation effects on phosphatase activities in milk. Dairy Sci. Abs. 24 : 1166.

Veringa, H. A. 1966. Sterilization of milk ionizing radiation. Dairy Sci. Abs. 29 : 1765.

Wallace, G. M. and K. R. Aiyar. 1969. Sulfhydryl and disulfide groups in casein. J. Dairy Res. 36 : 115-23.

Wertheim, J. H., B. E. Proctor and S. A. Goldblith. 1956. Radiation preservation of milk and milk products. IV. Radiation induced browning and some related chemical changes in milk. J. Airy Sci. 39 : 1236-46.

Wertheim, J. H., R. N. Roychoudhury, J. Hoff, S. A. Goldblith and B. E. Proctor. 1957. Irradiation preservation of milk and milk products. J. Agr. Food Chem. 5 : 944-50.

Wood, F. W. 1968. Unpublished data. Department of Food Science, University of Alberta.

Worseck, M. 1961. Studies on the inactivation of milk microbes with cobalt-irradiation. Dairy Sci. Abs. 23 : 2312.

Yoshino, U., H. K. Wilson and E. O. Herreid. 1962. Amperometric titration of sulfhydryl and disulfide groups in milk proteins. J. Dairy Sci. 45 : 1459-64.

Zweig, G. and J. R. Block. 1953. The effect of heat treatment on the sulfhydryl groups in skimmilk and non-fat dry milk. J. Dairy Sci. 36 : 427-36.